DEMOGRAPHICS AND TELOMERE DYNAMICS OF HIBERNATING ARCTIC GROUND SQUIRRELS (*UROCITELLUS PARRYII*)

By

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Abstract

Aging is the complex process by which an organism loses functional integrity over time. Several measurable contributors to or components of the aging process have been identified, one of which is telomere length. Telomeres are the repetitive, nucleoprotein structures located at the ends of linear chromosomes. In general, telomeres shorten over time and when exposed to damaging reactive oxygen species (highly unstable molecules released as a byproduct of cellular respiration). Organisms that have unique physiologies, in addition to those that live longer than otherwise predicted, have recently inspired comparative telomere dynamics studies. Hibernating mammals, which exhibit both heterothermy and long lifespan, have served as models for these new investigations into telomere length dynamics. Several studies over the past decade have measured the effects of torpor (the period of hibernation characterized by extremely low metabolic rates and body temperature) and arousal (from torpor; a brief return to euthermic or high levels of body temperature) on telomere length change in hibernators. This body of work demonstrated that telomere length is preserved across hibernation seasons (likely due to the majority of hibernation spent in torpor), and any telomere shortening that does occur is correlated with arousal frequency. However, all telomere-hibernator studies to date have focused on hibernators from temperate regions and on DNA from a peripheral tissue (either buccal cells or skin tissue). Arctic ground squirrels, the northernmost hibernator and ground squirrel species, are appropriate model candidates to expand the diversity of research in hibernator telomere dynamics, as they remain thermogenic during torpor to defend a viable body temperature against subfreezing ambient temperatures. Maintaining high metabolic rates to support thermogenesis throughout torpor—and over arousals—may lead to increased telomere attrition in this species compared to other hibernators adapted to milder climates. This thesis begins with basic arctic ground squirrel demographics from two well-studied populations in Arctic Alaska. I report that (female) arctic ground squirrels appear to be similarly long-lived as other hibernating species, and that sex-specific differences in lifespan may be driven by behavioral differences between males and females. I also present results from a study comparable to those performed in temperate hibernators: I measured telomere length in free-living arctic ground squirrels across hibernation and age groups and found that, as in temperate hibernators, telomere length (in ear tissue) is maintained across hibernation. Expanding upon single-tissue telomere studies, I also measured telomere length in brown adipose tissue (the tissue responsible for non-shivering thermogenesis for heat generation during torpor and at arousal initiation), liver, and heart in captive arctic ground squirrels and found that telomeres

shortened dramatically in brown adipose tissue only. Overall, this work identifies arctic ground squirrels as capable of maintaining cellular integrity (as measured via telomere length) and of reaching surprising longevity in the face of extreme environmental conditions.

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General Introduction

Aging can be defined as the functional decline experienced by an organism over time (López-Otín et al., 2013). How and why organisms age has been intensely investigated over the last few decades, in part inspired by the discovery in the early 1980s of mutations that can increase the longevity of *Caenorhabditis elegans* (Klass, 1983). Over the following decades, several theories of aging have emerged, which fall into two main categories: those arguing that aging is preprogrammed—as exemplified by organisms that senesce quickly after reaching sexual maturation and/or undergo reproduction (Skulachev, 2012)—and those proposing that aging is due to damage accumulation (Kirkwood, 2005; Sergiev et al., 2015; da Costa et al., 2016). Theories within the latter category are united by the fact that, over time, macromolecule and organelle functionality is reduced (Balaban et al., 2005), repair pathways break down (Lombard et al., 2005), and undegradable metabolic byproducts accumulate within organisms (reviewed in Sergiev et al., 2015). Underlying both preprogrammed and damage theories are several "hallmarks" of aging, including stem cell exhaustion, cellular senescence, genomic instability, and telomere attrition, among others (Barja, 2004; Kirkwood, 2005; Armanios et al., 2009; López-Otín et al., 2013; Finkel, 2015).

Telomeres are the repetitive, nucleoprotein sequences that cap linear (eukaryotic) chromosomes (Blackburn, 1991; Allsopp et al., 1992; Blackburn, 2000; Monaghan et al., 2018). These specialized, non-coding sequences are ancient and highly conserved (TTAGGG/AATCCC in all vertebrates, and similar sequences in invertebrates), indicating the essentiality of their role in maintaining chromosome integrity (Meyne et al., 1989; Campisi et al., 2001; Monaghan et al., 2018). Telomeres solve two "problems" that are characteristic of linear chromosomes: the end-*replication* problem, whereby nucleotides at the 5'-end of the telomere are lost with each round of cell division (Harley et al., 1990; Levy et al., 1992), and the end-*protection* problem, whereby the ends of linear chromosomes appear as targets to DNA repair machinery (de Lange, 2009). As telomeres are non-coding and located at the ends of chromosomes, they act as buffers to protect interstitial coding DNA from disruption during cell division, thus resolving the end-replication problem. The unique telomeric structure mediates the end-protection problem: the very end of the telomere is single-stranded (in humans, the single-stranded, 3'-overhang is 35–600 nucleotides in length; Sfeir et al., 2005), and this end invades an upstream portion of the double-stranded telomere sequence to create a t-loop (de Lange, 2005; Schmutz and de Lange, 2016). In mammals, this t-loop structure is formed and maintained by a six-protein complex

known as shelterin (de Lange, 2005). "Hiding" the chromosome end prevents DNA damage signaling from reaching repair mechanisms and also prevents chromosome-chromosome fusion (Monaghan, 2010).

Although telomeres do shorten with each round of cell division, replication is not the only, nor the most significant, contributor to telomere attrition. Another mechanism by which telomeres can shorten is through disruption of the telomeric sequence via reactive oxygen species (ROS; von Zglinicki et al., 2000; von Zglinicki, 2002; Houben et al., 2008; Monaghan et al., 2009). ROS are highly unstable molecules that are produced exogenously (via UV radiation, environmental pollutants, smoking, etc.; reviewed in Kohen and Nyska, 2002) or endogenously through electron leak and reactions with molecular oxygen during cellular respiration (Turrens, 2003; Jastroch et al., 2010). The interaction of ROS and a telomeric guanine creates a lesion that may be removed by DNA repair machinery, ultimately creating a single-strand break (Fouquerel et al., 2016; Barnes et al., 2018). When DNA polymerase encounters this break over the subsequent round of DNA replication, replication may be disrupted and the remainder of the telomere is left unreplicated, leading to pronounced shortening (Oikawa and Kawanishi, 1999; von Zglinicki et al., 2000; Kawanishi and Oikawa, 2004).

Telomeres can also lengthen. The holoenzyme telomerase is comprised of reverse transcriptase catalytic proteins and telomerase RNA (Chan and Blackburn, 2004; Blackburn, 2005; Shay and Wright, 2019). When telomerase attaches to a telomere, the RNA component acts a template for adding the correct nucleotides to the end of the telomere, thereby extending the 3'-end. DNA polymerase III can then complete the telomere extension by adding nucleotides to the 5'-end (the 3'-overhang is maintained in this process; Vega et al., 2003). In most human somatic cells, telomerase maintains very low levels of activity, which is thought to be a cancer-preventative mechanism: overactive telomerase is detected in 80-90% of cancers (Campisi et al., 2001; Chan and Blackburn, 2004; Blackburn et al., 2015). In other, smaller-bodied animals, such as rodents, somatic cells often maintain higher levels telomerase activity on a tissue-specific basis (Seluanov et al., 2007; Gorbunova and Seluanov, 2009).

Although it has not been firmly established that telomere attrition is a cause of aging (but see Armanios et al., 2009), it has long been understood that telomeres shorten with time and with each cell division. Early studies of human fibroblast cells revealed that telomeres shorten as cells age (Harley et al., 1990; Allsopp et al., 1992; Levy et al., 1992). Two decades

later, telomere attrition has been defined as a hallmark of aging (Kirkwood, 2005; López-Otín et al., 2013), and telomere length has been extensively used as a biomarker to predict longevity (Ujvari and Madsen, 2009; Monaghan, 2010; Heidinger et al., 2012; Dantzer and Fletcher, 2015; Foley et al., 2018) and to quantify an organism's accrued oxidative damage (Monaghan and Haussmann, 2006; Cattan et al., 2008; Houben et al., 2008; Reichert and Stier, 2017).

Understanding telomere dynamics via potential ROS-mediated shortening *in vivo* has inspired recent studies into the effects of hibernation physiology on telomere length (Turbill et al., 2012; Turbill et al., 2013; Giroud et al., 2014; Hoelzl et al., 2016). Hibernation in small mammals is characterized by two metabolic states: torpor and periodic arousal (Carey et al., 2003; Ruf and Geiser, 2015; Staples, 2016). Torpor is an extended (~3 week) period of dramatically suppressed metabolism and body temperature (T_b). During this time, average heart rate across hibernators slows from 155 to 9 beats min^{-1} (Zatzman, 1984), respiration rates decrease to 2% of basal levels (Boyer and Barnes, 1999), and cell division is arrested (Kruman et al., 1988; Wu and Storey, 2012). In contrast, an arousal is a brief (~15 hour) return to euthermic levels of T_b and metabolism, and is associated with the occurrence of oxidative damage and, in some tissues, antioxidant influx (Tøien et al., 2001; Orr et al., 2009; Wei et al., 2018). Arousal episodes occur regularly throughout hibernation and are thought to support cellular maintenance and the restoration of metabolic or neurobiological homeostasis (Nelson et al., 2010; Epperson et al., 2011), although a full understanding of their role has not yet been elucidated (reviewed in Staples, 2016).

Studies of hibernator telomere dynamics have been inspired both by this interplay between torpor—during which time telomeres appear to be maintained (Turbill et al., 2012; Turbill et al., 2013)—and arousals—whose frequency is correlated with any telomere shortening that does occur (Giroud et al., 2014; Hoelzl et al., 2016)—and with hibernator lifespan. Small hibernating mammals have longer maximum recorded lifespans compared to similar-sized non-hibernators: for a 50-g hibernating species, maximum lifespan is about 50% greater than for a 50-g non-hibernator (Turbill et al., 2011). This longevity is supported by comparably high annual survival (due to winter sequestration and subsequent protection from predators) and is associated with slow life histories (Turbill et al., 2011). In addition, there is evidence to suggest that time spent at low metabolic rates and T_b may extend lifespan (Lyman et al., 1981; Wu and Storey, 2016). Studies have shown that frequent torpor use maintains telomere length (Turbill et al., 2012) and that, overall, hibernation slows telomere attrition (Turbill et al., 2013; Giroud et

al., 2014; Hoelzl et al., 2016), suggesting that torpor supports somatic maintenance and—at least in terms of telomere length—retards aging.

Past work on hibernator telomeres has laid a foundation for exploring how these aging/stress biomarkers change in other hibernating species, including those that must stay thermogenic during torpor. All previous work sampled from temperate animals that defended a torpor T_b at above-freezing ambient temperatures (T_as). If a small mammal is hibernating at a T_a above the freezing point of its tissues, its mean torpid metabolic rate will drop to 2-6% of its basal metabolic rate (Boyer and Barnes, 1999; Ruf and Geiser, 2015) and its T_b will approach T_a. It is possible for some small hibernating mammals to defend their T_b against subfreezing T_a, and to do so, they must remain thermogenic during torpor to maintain a viable T_b (Geiser and Kenagy, 1988; Buck and Barnes, 2000; Karpovich et al., 2009).

One hibernator that defends its T_b against some of the coldest and longest winters on Earth is the arctic ground squirrel (AGS; *Urocitellus parryii*), whose distribution ranges from the Yukon and British Columbia to Siberia and Kamchatka (McLean, 2018). In the northern parts of its range, winter mean soil temperature is -10°C, with a minimum of -26°C (Buck and Barnes, 1999). AGS can supercool their core T_b to as low as -2.9°C (with a mean torpid core T_b of -1.7°C; Lee et al., 2015) while maintaining their ability to spontaneously arouse to euthermia (Barnes, 1989), and female AGS may hibernate and remain belowground as long as eight months (males hibernate for approximately six months; Sheriff et al., 2011). To support a ~25°C gradient between T_b and T_a, torpid AGS can raise their metabolic rates by 36-fold during torpor (Richter et al., 2015), and to arouse at such low T_a, AGS must increase their metabolic rate to ~176% of basal rates (Karpovich et al., 2009). High metabolic rates throughout hibernation in AGS contrast with lower rates experienced by animals hibernating at milder T_a. ROS that may be produced during periods of high metabolic activity in hibernating AGS could subsequently shorten telomeres to a greater degree or at a higher rate than in temperate hibernators. The maximum lifespan of AGS is currently unknown, and perhaps this hibernator lives a shorter life than other hibernating species due to this faster pace of life experienced over extended, subfreezing winters.

It has been established that, in many species, lifespan and telomere length are linked (Monaghan and Haussmann, 2006; Heidinger et al., 2012; Dantzer and Fletcher, 2015). The goals of my research were to determine the maximum lifespan and survival estimates for AGS

and to provide the first account for how telomeres change across the hibernation season and with age in this well-studied hibernating species. Chapter 1 reports basic demographics for two populations of AGS north of the Brooks Range in Arctic Alaska. As these populations have been monitored and sampled extensively for nearly 30 years and work is currently ongoing, it is important to quantify AGS longevity, survival estimates, and how biologging devices impact survival in moving forward with future physiological, ecological, and behavioral work in these animals. Chapter 2 examines telomere length change over hibernation and across age cohorts in free-living AGS, and concludes that telomere length appears to be preserved across hibernation in a peripheral tissue, perhaps indicating that cellular integrity is maintained in AGS in spite of high thermogenic demands during torpor and arousal at subzero ambient temperatures. Finally, Chapter 3 moves from peripheral tissues to those that are more implicated in AGS arousal thermogenics. Using DNA extracted from brown adipose tissue (BAT), liver, and heart, we measured tissue-specific telomere length from AGS sampled at mid-hibernation and late hibernation. BAT telomeres were significantly shorter at late hibernation than at mid-hibernation, suggesting that ROS released upon arousal in this tissue are degrading telomeres. This third study is an important evolution of past telomere-hibernator studies—including the study discussed in Chapter 2 of this thesis—that relied solely on peripheral tissues (buccal and/or skin cells) to understand patterns of telomere length change throughout hibernation.

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Chapter 1: Effects of Age, Sex, and Biologging on Survival of Arctic Ground Squirrels[1]

1.1 Abstract

Hibernation confers longer lifespan than expected: hibernating mammals live, on average, 15% longer than non-hibernators of equivalent mass. Although we have a basic understanding of how hibernation correlates with lifespan, what is less understood is how this varies within hibernating species on a sex-specific basis. In polygynous mammalian species, males have shorter lifespans than females due to reproductive competition, and in hibernating mammals, this could also be driven by differences in hibernation duration. Arctic ground squirrels (AGS; *Urocitellus parryii*) differ markedly in sex-specific hibernation durations: female AGS hibernate about 30% longer than males. However, basic, sex-specific demographics are unknown for AGS. Using Cormack-Jolly-Seber modeling, we used 13 years of mark-recapture data from two populations of AGS in Arctic Alaska to quantify lifespan, apparent annual survival, and the effects of deployed biologgers—which have been used extensively in these populations to study physiology and behavior—on survival. We report here that females can outlive males by four years and have higher annual survival. We also show that surgery associated with biologger implantation has a slightly negative impact on survival. Quantifying basic AGS demographics from these two well-studied populations informs how changes in climate or ecology of the region may influence population processes in this species.

[1] Wilbur, S.M., Deane, C.E., Barnes, B.M., Breed, G.A., Buck, C.L., and Williams, C.T. Effects of age, sex, and biologging on survival of arctic ground squirrels (*Urocitellus parryii*). To be submitted to *Canadian Journal of Zoology*.

1.2 Introduction

Hibernation is a strategy that some mammals use to survive predictable periods of food unavailability, often during winter months (Geiser 1998; Staples 2016). The long periods of inactivity characteristic of hibernation usually take place in a burrow or cave, which protects the animal from predation and in turn supports high overwinter survival (Turbill et al. 2011). Turbill et al. (2011) calculated that hibernating mammals have, on average, a 15% higher annual survival rate than non-hibernators of equivalent mass, and that hibernators live longer than non-hibernators (for example, a 50 g hibernating mammal was predicted to have a maximum lifespan that would exceed the lifespan of its non-hibernating counterpart by 2.8 years). The ability of a hibernator to reduce its metabolic rate for extended periods—thereby decreasing its overall pace of life—may also contribute to increased lifespan (Lyman et al. 1981; Wu and Storey 2016).

Although we have a basic understanding of how hibernation use correlates with survival and lifespan, sex-specific differences in these demographic parameters have not been studied extensively across a wide range of hibernating species. Many vertebrates—including humans—exhibit sexual dimorphism in survival and longevity, which may be driven by differences in how the sexes optimize the fundamental tradeoff between reproduction and survival (Clutton-Brock and Parker 1992; Bonduriansky et al. 2008; Maklakov and Lummaa 2013). In polygynous vertebrates, males have higher annual rates of mortality than females due to intraspecies aggression during reproductive competition (Clutton-Brock and Isvaran 2007). In hibernating species, sex-specific differences in hibernation phenology (when immergence/emergence occurs and/or length of hibernation) and aboveground behavior may drive differences in survival and maximum lifespan between males and females.

Arctic ground squirrels (AGS; *Urocitellus parryii*) are the northernmost and largest ground squirrel species (McLean 2018). The sexes differ in their hibernation timing: in northern Alaska, adult females enter hibernation in August/early September and first emerge beginning in late April, while adult males hibernate for a shorter period, entering their hibernacula by early October and emerging in early to mid-April (Carl 1971; Sheriff et al. 2011); thus, AGS females hibernate approximately 30% longer than males each season. The sexes also differ in aboveground activity patterns, with males dispersing prior to or after their first hibernation season (Byrom and Krebs 1999; Buck and Barnes 1999a) and engaging in aggressive male-male conflicts in the spring (to defend territories; Lacey and Wieczorek 2001) and fall (to defend food

caches; Buck and Barnes 2003; Richter et al. 2017). These differences in hibernation parameters and behavior between male and female AGS are more pronounced than sex differences in other hibernating ground squirrel species (e.g. Wang 1979; Michener 1983; Young 1990; Rieger 1996; Healy et al. 2012).

Since 1990, a population of AGS near the Toolik Field Station in Arctic Alaska (68°38′N, 149°38′W) has been monitored and sampled annually for physiological and ecological studies through the University of Alaska Fairbanks (e.g. Buck and Barnes 1999a; Buck and Barnes 1999b; Buck and Barnes 2000; Sheriff et al. 2011; Wilsterman et al. 2015; Williams et al. 2017). Beginning in 2006, an additional population of AGS—20 km south of the Toolik Field Station and east of the Dalton Highway near the Atigun River (68°27′N, 149°21′W)—was incorporated into this work; both sites have experienced yearly trapping efforts since 2006. To understand AGS hibernation physiology/parameters, aboveground activity, circadian/circannual rhythms, and other aspects of their biology, some marked animals were provided one (or several) biologging devices, including collar-mounted light/temperature loggers and accelerometers, and implanted temperature loggers. While biologging can provide critically important information on the physiology, behavior, and life history of free-living organisms, it is important to consider whether the biologgers impact survival, or other parameters, in these populations (Wilson et al. 2015; Bodey et al. 2018). Furthermore, while this work has provided us detailed knowledge on AGS physiology and ecology, very little is known regarding basic demographic parameters such as annual survival and sex-dependent maximum lifespan. Here, to estimate AGS survival and detectability, we capitalize on mark and recapture efforts that occurred alongside sampling for specific studies. These data allow us to address sex-specific differences in reproductive requirements and hibernation duration on survival and longevity. We predicted that 1) AGS would have comparable maximum lifespans—based on mass—to other hibernating species, and 2) shorter hibernation seasons and risky behavior exhibited by AGS males would lead to reduced lifespan and survival in this sex. We also used these data to examine whether surgical implantation or collaring of AGS negatively impacts survival.

1.3 Materials and Methods

1.3.1 Study Species and Area

AGS are semi-fossorial rodents with a Holarctic distribution that includes montane and Arctic Alaska (McLean 2018). All AGS used in this study were captured adjacent to the Dalton Highway near the Atigun River (hereafter: Atigun; elevation 812 m) or near Toolik Lake

(hereafter: Toolik; elevation 719 m) in northern Alaska, USA. The Atigun site is approximately 27 ha, while the Toolik site is approximately 85 ha. Population density at Atigun is higher than at Toolik, likely due to the prevalence of sandy soils—which are highly suitable for burrowing—at Atigun. See Sheriff et al. (2011) for additional site characteristics.

1.3.2 Trapping Effort

We used a long-term mark and recapture dataset from Atigun and Toolik—developed as part of a series of studies investigating the physiology, life history, and phenology of AGS—to estimate demographic parameters. Between 2006 and 2018, recaptures were consistently recorded for each age/sex group (save for juveniles of both sexes in 2016) at both sites. The number of unique individuals marked within this date range was 1127 (270 adult females, 349 adult males, 235 juvenile females, and 273 juvenile males). This 13-year data set was used for determining male and female maximum lifespans and for a mark-recapture analysis to quantify the effects of age, sex, site, and biologger deployment on survival and detection in AGS. (Note: although trapping also occurred prior to 2006 at Toolik only, we excluded this data from our analyses due to low and inconsistent trapping effort across years).

To capture animals, we used Tomahawk Live Traps (14×14×40 cm; Tomahawk Live Trap Co.; Tomahawk, Wisconsin, USA) baited with carrot. Traps were generally set out between 0900 and 1700, weather permitting (AGS are sensitive to unfavorable environmental conditions and may stay underground in inclement weather; Long et al. 2005; Williams et al. 2016). In general, trapping efforts were variable across years. Although effort over a particular summer was often focused on marking or recapturing squirrels for a specific project, effort was made to mark newly encountered adults and to record the first annual recapture of animals; however, juveniles were frequently released without marking. Subsequent recaptures within a year were not always recorded. As many AGS included in the dataset were fitted with biologgers, these individuals were preferentially chosen from the center of both sites in an attempt to prevent losing these animals to dispersal. Additionally, relative to animals first captured, recaptured animals were preferentially equipped with biologgers.

1.3.3 Age Designation

Age was determined and designated as follows: upon initial capture, young-of-the-year animals were distinguished from yearlings/adults by their relatively small mass and unmolted pelage. Because juveniles disperse in the fall, we assumed any unmarked adult AGS captured on

the study site was a yearling that had dispersed the fall prior and thus designated it as one year old. At first capture, animals were given an ear tag (Monel No. 1005-1 tag; National Band and Tag Co.; Newport, Kentucky, USA). Beginning in 2014, nearly all animals (82-100%, depending on year) received a passive integrated transponder (PIT) tag (Avid Identification Systems, Inc; Norco, California, USA) upon first capture. Prior to 2014, most animals received a PIT tag when first receiving a collar or surgery (to implant/explant a temperature logger).

1.3.4 Biologgers

Several biologging devices were deployed on AGS between 2006-2018: light/temperature loggers and accelerometers were deployed on collars, and temperature loggers were implanted into the peritoneal cavity. Light/temperature loggers were used to determine when AGS emerged from their burrows or were belowground (based on exposure to light), as well as when animals were torpid/aroused/active (based on body temperature; Williams et al. 2016). Accelerometers were used for monitoring daily patterns of movement, while implanted temperature loggers were used to measure patterns of core body temperature during both the active and hibernation seasons.

Three types of light/temperature loggers were deployed on collars: BAS model MK7290 light loggers (Biotrack Ltd; Dorset, UK), which record light levels every two minutes; Intigeo-C56 light loggers (1 g; Migrate Technology Ltd; Cambridge, UK), which record light every minute and then save the highest measured value per five-minute interval; and GeoLT light loggers (8 g; Earth and Ocean Technologies; Kiel, Germany). The collared-deployed accelerometers were axy-3 and axy-4 loggers (less than 3 g; TechnoSmArt Europe Srl; Colleverde, Italy). Two types of abdominal, temperature-sensitive data loggers were implanted/explanted between 2006-2018: modified TidBiTs (StowAway model TBICU32-05þ44; 12-14 g; Onset Computer Corporation; Bourne, Massachusetts, USA) and iButtons (model DS1922L; ~5 g; Maxim Integrated Products; Sunnydale, CA, USA), programmed to record temperature every 10 minutes. For details on surgical procedures, see Long et al. (2007).

1.3.5 Maximum Observed Lifespan and Mark-Recapture Analysis

We determined maximum observed lifespan for both male and female AGS by quantifying the ages of the oldest individuals recaptured between 2006 and 2018. To determine the effects of known age (juvenile vs. adult at first capture), sex, site, and biologger deployment on apparent annual survival probability (ϕ) and detection probability (p), we used the Cormack-

Jolly-Seber (CJS) model (Cormack 1964; Jolly 1965; Seber 1965) implemented in Program MARK (White and Burnham 1999). MARK analyses were performed in R (R Core Team 2019) and accessed via the RMark package (Laake 2013). ϕ is the probability that an AGS alive in year i remained available for re-sighting until year $i+1$, while p is the probability that an AGS alive in year i was observed in that year.

Collared devices were deployed on 137 animals, temperature logger surgeries were performed on 319 animals, and 635 animals received PIT tags. Biologger and PIT tag deployment were entered into MARK as time-varying covariates (by year), with a "1" indicating deployment (or removal, in the case of a surgery for removing a temperature logger) and a "0" indicating removal or absence (once an animal received a PIT tag, "1" was entered for each subsequent year).

Each candidate model for ϕ contained one of three covariates: known age, sex, or age/sex (e.g. adult female). In addition to one of these three covariates, all possible combinations of the covariates site, collar, surgery, and PIT tag were included, following the candidate model strategy proposed by Doherty et al. (2012). A high positive effect of surgery on estimates for ϕ of each age/sex group led us to specify the time of surgery (fall or spring) as two additional covariates (the "general" surgery covariate was then dropped from candidate models). We also included a fall encounter covariate in each candidate model, as we predicted fall-encountered animals would have higher survival and to better account for the positive effect of fall surgery on estimates of ϕ for each age/sex group. Candidate models for p followed a similar pattern but did not include covariates for collar, fall/spring surgery, and fall encounter, as initial model selection indicated that too few AGS had received collars or surgery or were encountered in the fall to provide meaningful estimates of these covariates for p.

We selected the best models from the candidate list of models using Akaike's information criterion adjusted for both over-dispersion and sample size ($QAIC_c$; Burnham and Anderson 2002). The $QAIC_c$ values for each model were obtained from Program MARK, and the model with the lowest $QAIC_c$ value we considered to be the best-supported. Differences in $QAIC_c$ values between the best model and every other ($\Delta QAIC_c$) were used to evaluate the plausibility of each model. For any model with a $\Delta QAIC_c$ value <2, there is substantial evidence that that model is the best model (Burnham and Anderson 2002). Therefore, we chose to report the five best-supported models (all with $\Delta QAIC_c$ values <2) and, as estimates were very similar across these

five models, we present estimates for the top three models only (see Table A1 in Appendix for a list of models with summed weight of 0.90).

As Program MARK provides parameter estimates on the logit scale, we back-transformed beta (ß) estimates and the associated standard error for each age/sex cohort covariate to obtain real parameter estimates (on the probability scale). We report ß estimates for the remaining covariates (collar, spring surgery, fall surgery, fall encounter, PIT tag, and site).

1.4 Results

1.4.1 Maximum Observed Lifespan

The oldest female AGS recaptured between 2006 and 2018 was ten years old, while the oldest male recaptured was six years old (Figure 1.1). The oldest female was recaptured at Atigun and the oldest male at Toolik (at Atigun, the oldest recaptured male was five years old, and at Toolik, the oldest recaptured female was seven years old).

1.4.2 Top Model Structures

For *ø*, age/sex and PIT were included in all five top models (Table 1.1). Fall and spring surgery were estimated in all models, save for the fifth model, which did not include spring surgery. Site was estimated only in the third-best model, and collar was estimated only in the fourth-best model. For *p*, all models included age/sex, site, and PIT, save for the second-best model, which estimated known age rather than age/sex.

1.4.3 Estimates for Apparent Annual Survival (*ø*)

Across the four age/sex groups (adult females, adult males, juvenile females, and juvenile males), adult females had the highest estimate for *ø* (Table 1.2). Adult male and juvenile female *ø* estimates were comparable, with a slightly lower *ø* in juvenile females. Juvenile male *ø* was the lowest of all age/sex groups. If an animal was encountered in the fall, *ø* was higher than animals not recorded as encountered in the fall (i.e. recorded as captured in spring or summer; Table 1.3). A spring surgery was slightly deleterious to *ø*, compared to animals that did not receive surgery in the spring, while a fall surgery reduced *ø* relative to animals encountered in the fall. PIT tagging had a slightly negative effect on *ø* (Table 1.3).

1.4.4 Estimates for Detection (p)

Among age/sex groups, p was highest for adult females (Table 1.2). The estimate for adult male p was somewhat lower than for adult females, and animals tagged as juveniles—particularly males—had lower estimates for p as yearlings (Table 1.2). PIT tagging increased p dramatically, while animals at Toolik were less likely to be detected than animals at Atigun (Table 1.3).

1.5 Discussion

AGS physiology, phenology, and behavior has been studied extensively for decades, yet basic demographic information—including lifespan, survival probability ($ø$), and the effect of biologging devices—has not yet been reported. Here we analyze sex-specific lifespan, $ø$, and the impact of biologgers on $ø$, determined from 13 years of AGS population monitoring data at two sites in Arctic Alaska. In general, we found that female lifespan is consistent with other hibernators, that males appear to live much shorter lives, and that biologgers—particularly body temperature loggers that were surgically implanted—somewhat negatively impact $ø$. We also determined that, consistent with other hibernating species, AGS encountered in the fall had higher $ø$ than AGS encountered during other periods of the active season.

Consistent with sex-specific lifespan differences in other rodent species (Clutton-Brock and Isvaran 2007; Barrett and Richardson 2011), female AGS have a longer maximum lifespan than males. When compared to hibernators of similar mass (male AGS weigh 450–1,000 g, with some reports of male weights above 1 kg, while females weigh about 10% less; McLean 2018), AGS males are rather shorter lived than other hibernators, while AGS female lifespan generally coincides with the lifespans of other similar-sized hibernators (Turbill et al. 2011). In terms of how AGS survival probability compares to other hibernators, female $ø$ estimated here is consistent with the mean of similarly-sized hibernators, while male AGS $ø$ is somewhat lower (Turbill et al. 2011). Also in agreement with results reported in Turbill et al. (2011) is our finding that overwinter $ø$ is high in AGS (as demonstrated by our fall encounter estimate).

It is not unusual that a female mammal would outlive a male: this is a pattern common to vertebrate species where the male competes with other males for reproductive opportunities [Clutton-Brock and Isvaran 2007; Maklakov and Lummaa 2013; alternatively, differences in lifespan may be due to sex-specific genetic causes (Tower and Arbeitman 2009) or to selection for longer life in the caregiving sex (Young et al. 2013)]. Indeed, the propensity of females to

outlive males is common across ground squirrel species, including Richardson's (*Urocitellus richardsonii*; Michener 1989), Townsend's (*Urocitellus townsendii*; Smith and Johnson 1985), and Uinta (*Urocitellus armatus*; Rieger 1996) ground squirrels. One behavioral aspect that differs between these species and AGS is the dissimilarity in hibernation duration between male and female AGS. Female AGS may hibernate as much as 30% longer than males, immerging into their hibernacula in August and emerging by late April, while males enter hibernation as late as October and emerge in early April (Carl 1971; Sheriff et al. 2011). In contrast, the duration of hibernation differs less between the sexes in other ground squirrel species. For example, the onset and termination of hibernation occur at similar times of the year in both sexes of thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*), and females only marginally outlive males (McCarley 1966). In Townsend's ground squirrels, females emerge from hibernation 1-2 weeks later than males and also appear to only slightly outlive males (Smith and Johnson 1985; immergence times were not reported). Michener (1989) reported that, in a population of Richardson's ground squirrels in southern Alberta, females outlived males by two years (six-year maximum lifespan for females; four-year for males). In another Richardson's ground squirrel study, Wang (1979) found that males enter hibernation a month after females [it appears that hibernation ends at a similar time (middle to late March) for males and females, but this was not stated explicitly]. Overall, sex-specific differences in hibernation duration and in lifespan in AGS are more pronounced than in other ground squirrel species.

Why might pronounced differences in hibernation duration support sex-specific differences in lifespan? Two shared characteristics of many (if not all) ground squirrel species is that males aggressively defend territories after spring emergence and that males disperse away from their natal burrows (Rongstad 1965; Dobson 1983; Michener 1983; Smith and Johnson 1985). These behaviors are also displayed by male AGS, in addition to the storing of a food cache and related agonistic behaviors. In the early fall, both juvenile and adult male AGS remain active and aboveground to store and defend a food cache to be consumed the following spring prior to emergence (Buck and Barnes 2003). This fall cache defense can become aggressive (Richter et al. 2017), a behavior that repeats upon emergence in mid-March when males stake out their territories prior to mating (Lacey and Wieczorek 2001); these interactions can be highly injurious or deadly (Batzli and Sobaski 1980). Furthermore, juvenile/yearling males tend to disperse away from their natal burrows both before and after their first hibernation season, while females remain close to kin (Byrom and Krebs 1999). Solitary males may be easier prey: Melchoir (1971) found that nearby AGS respond to an individual's alarm calls for predators, thus

providing indirect protection to any unwary animal. While these risky behaviors are seen across hibernating sciurids, if AGS males are engaging in aggressive disputes and dispersing while females are still hibernating and protected from predation for at least a month in both fall and spring, it follows that AGS males may experience higher mortality and, ultimately, shorter lifespan.

In addition to quantifying basic survival estimates, we also analyzed how biologger deployment impacted AGS ϕ. A detailed review by Murray and Fuller (2000) investigated the effects of marking and biologger deployment on vertebrate biology and found that tracking an animal's behavior with devices can have a wide range of effects on that animal's behavior and/or survival. Using radiotransmitters (collar-deployed) as an example, studies of the effects of radiotransmitter deployment on meadow voles (*Microtus pennsylvanicus*) showed varying results: one study reported no effect of deployment on behavior (Berteaux et al. 1996), while others showed negative effects on activity (Hamley and Fall 1975), weight and survival (Webster and Brooks 1980), and social relationships (Berteaux et al. 1994). In larger mammals, the effects of collar-deployment also vary and appear to be driven by collar weight. For example, in domestic cats, collars below 2% of the animal's body mass had no effect on ranging distance, while larger collars (3% of body mass) reduced home-range size (Coughlin and van Heezik 2015). In plains zebras (*Equus burchelli antiquorum*), slightly heavier GPS collars (0.6 vs 0.4% of body mass) reduced travel rate by ~50% and interfered with grazing behavior; both types of collar were within acceptable weight limits (Brooks et al. 2008). For AGS in our two populations, collars (devices that includes an accelerometer, a light/temperature logger, or both) had little impact on estimates of ϕ, demonstrated by the fact that collar appeared only in the fourth-best model out of our top five. This is likely due to the minimally-invasive nature of the collars, which are light in mass (~5 g, or 1% of body mass of a 500-g AGS) and are deployed with the logger epoxied to a continuous section of tubing to prevent neck abrasion (Williams et al. 2014).

In contrast to collar deployment, surgeries to implant or remove body temperature loggers are more invasive (for our procedure details, see Long et al. 2007). We found a slightly negative effect of both spring and fall surgeries on AGS ϕ (fall surgery was actually estimated as a positive effect, but relative to the effect estimated for fall encounter, fall surgery was less positive). While fairly recent studies have investigated the effects of implanted biologgers on circadian rhythms (Leon et al. 2014) and running speed (Rojas et al. 2010) in small mammals,

studies that have investigated the effects of peritoneal implants on small mammal survival are scarce and somewhat outdated (e.g. Smith et al. 1980; Eagle et al. 1984; Van Vuren 1989). Furthermore, these studies did not use robust mark-recapture methods to test for the effect of implantation surgery on survival. Nevertheless, these few studies did not find a negative effect of implantation on survival, and the most common cause of mortality that did occur was from infection following surgery. It is possible that AGS from our populations are experiencing post-surgery infection, which may be leading to decreased ϕ. Alternatively, animals that have recently received a surgery may be more susceptible to predators or wounding by conspecifics during agonistic interactions (Batzli and Sobaski 1980; Lacey and Wieczorek 2001). Additionally, animals that are given surgeries are temporarily (<24 hours) removed from the population, which may carry costs to reestablishing their territories upon reintroduction.

The slightly negative effect of PIT tagging on ϕ was initially surprising. In contrast to the number of studies that have investigated the effects of implantation surgery on survival in small mammals, numerous studies have quantified the effect of PIT tagging on survival (e.g. Braude and Ciszek 1998; Ellison et al. 2007; Rigby et al. 2012). Almost without exception, this body of work has not discovered a negative effect of PIT tagging on survival. Perhaps in our populations of AGS, there is a confounding temporal effect—such as more PIT tagging occurring in spring compared to fall—that is creating this seemingly negative effect. PIT tagging also has a highly positive effect on detection, likely driven by the desire for researchers to recover animals with devices: PIT tagging is also confounded with the deployment of collars and body temperature loggers. Site also exhibited a large significant effect on detection probability—with animals less likely to be detected at Toolik—which may reflect more a more intensive and consistent trapping effort at Atigun.

Overall, we show that female AGS are relatively long-lived, and suggest that the four-year difference in lifespan between males and females is, in part, driven by a shorter hibernation period exhibited by males. Biologger deployment—particularly from surgeries—appears to have a slightly negative impact on ϕ. As physiological, phenological, and behavioral work in these AGS populations is current and ongoing, it is critical for personnel to be properly trained in surgical procedures to support optimum health and post-implantation survival. Our results regarding the effect of surgery on survival contrast with a meta-analysis in birds demonstrating that biologger implantation may be preferable to external attachment (White et al. 2013). This study by White et al. (2013), in addition to a recent opinion article advocating for biologger

implantation over external attachment (Forin-Wiart et al. 2018), indicate that further research on this topic in small mammals is warranted. Our findings presented here provide basic, yet important, demographic information to support informed AGS studies into the future.

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1.8 Figures

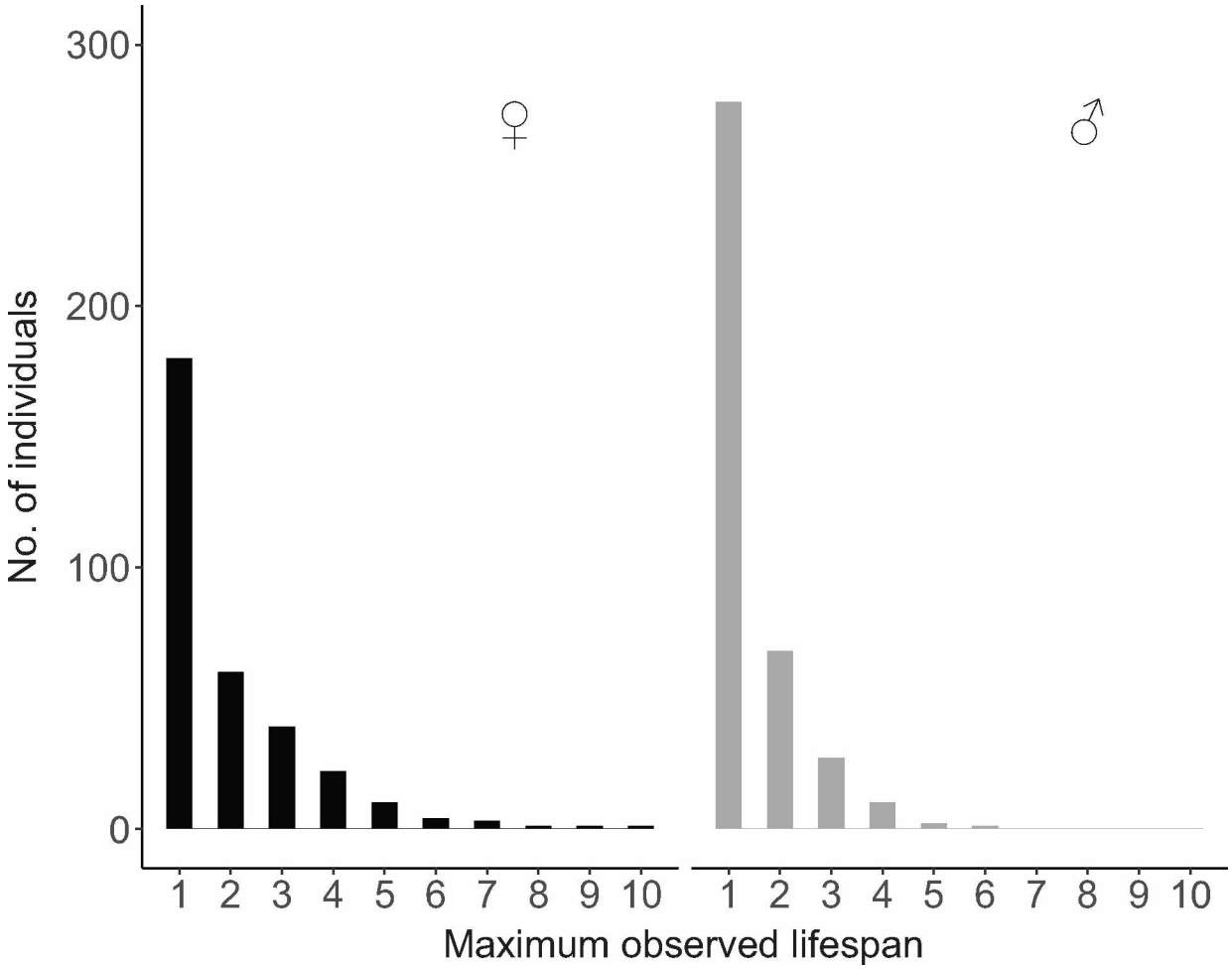

Figure 1.1. Histograms (female, left; male, right) of number of adult individuals per maximum observed lifespan. Data from 2006-2018 at two sites (East Atigun and Toolik).

1.9 Tables

Table 1.1. Top five model structures. Models reported here have a $\Delta QAIC_c$ <2. See Table A1 in Appendix for models whose summed weight = 0.90.

Covariates related to apparent annual survival	Covariates related to detectability	No. of parameters	$QAIC_c$		
			$\Delta QAIC_c$	Weight	Deviance
Age/Sex + PIT + Spring surgery + Fall surgery + Fall encounter	Age/Sex + Site + PIT	14	0.00	0.15	2113.15
Age/Sex + PIT + Spring surgery + Fall surgery + Fall encounter	Known age + Site + PIT	12	1.32	0.08	2118.53
Age/Sex + Site + PIT + Spring surgery + Fall surgery + Fall encounter	Age/Sex + Site + PIT	15	1.44	0.07	2112.55
Age/Sex + Collar + PIT + Spring surgery + Fall surgery + Fall encounter	Age/Sex + Site + PIT	15	1.53	0.07	2112.64
Age/Sex + PIT + Spring surgery + Fall encounter	Age/Sex + Site + PIT	13	1.93	0.06	2117.11

Table 1.2. Apparent annual survival (*ø*) and detection (*p*) estimates for age/sex cohorts. Estimate order within each cell matches model order as presented in Table 1.1. Estimates are reported on the real (probability) scale (±SE). Dashes (—) indicate that an estimate was not included in the corresponding model. Estimates reflect *ø* and *p* for Atigun animals.

Age/sex	Survival probability (*ø*)	Detection probability (*p*)
Adult females	0.451±0.040 0.448±0.040 0.454±0.040	0.670±0.070 — 0.671±0.070
Adult males	0.263±0.036 0.250±0.034 0.266±0.036	0.557±0.092 — 0.554±0.092
Juvenile females	0.203±0.036 0.211±0.037 0.205±0.036	0.355±0.090 — 0.353±0.089
Juvenile males	0.143±0.032 0.121±.026 0.144±0.032	0.153±0.061 — 0.153±0.061
Known age: adult	—	— 0.641±0.065 —
Known age: juvenile	—	— 0.272±0.066 —

Table 1.3. Apparent annual survival (*ø*) and detection (*p*) estimates for additional covariates. Beta (ß) estimates±SE are reported here. Estimate order within each cell matches model order as presented in Table 1.1. Dashes (—) indicate that an estimate was not included in the corresponding model.

Covariate	Beta (ß) estimate for survival (*ø*)	Beta (ß) estimate for detection (*p*)
PIT tag	-0.355±0.156 -0.319±0.156 -0.340±0.157	3.889±0.379 3.791±0.376 3.844±0.385
Collar	—	—
Spring surgery	-0.406±0.166 -0.411±0.167 -0.393±0.167	—
Fall surgery	0.354±0.181 0.359±0.181 0.380±0.183	—
Fall encounter	1.470±0.150 1.477±0.149 1.467±0.149	—
Toolik	— — -0.111±0.143	-1.850±0.324 -1.856±0.324 -1.735±0.360

Chapter 2: Telomere Dynamics in Free-Living Arctic Ground Squirrels[2]

2.1 Abstract

Telomeres—the dynamic ends of linear chromosomes—shorten with each cell division and in response to reactive oxygen species; thus, telomere length can be used as a marker of both an organism's predicted longevity and accrued oxidative damage. Metabolic depression in small mammalian hibernators is hypothesized to influence telomere dynamics: telomeres are generally maintained across hibernation but the degree of telomere shortening that does occur is correlated with the frequency of arousals to euthermia during hibernation. However, all previous telomere-hibernation work has been performed on temperate hibernators, which maintain very low metabolic rates throughout torpor bouts. In contrast, arctic ground squirrels (AGS; *Urocitellus parryii*) in Northern Alaska experience ambient conditions of prolonged subzero temperatures throughout hibernation, which forces animals to be thermogenic during torpor and increases metabolic rate during arousals. We examined whether relative telomere length (RTL) in ear tissue of free-living AGS shorten across hibernation or with age. We found no difference between pre- and post-hibernation RTL and were unable to detect an effect of age, although our ability to interpret age results was limited by both small sample sizes and a narrow range of ages. Our results suggest that increased costs of torpor and arousal at subzero hibernacula temperatures do not result in shorter telomere length—at least in ear tissue—in hibernating AGS.

[2] Wilbur, S.M., Kitaysky, A.S., Barnes, B.M., and Williams, C.T. Telomere dynamics in free-living arctic ground squirrels (*Urocitellus parryii*). To be submitted to *Canadian Journal of Zoology*.

2.2 Introduction

Mammalian hibernators lower their metabolism and body temperature (T_b) during seasons of resource scarcity (Geiser 1998; Staples 2016), and reduced metabolic rates throughout hibernation are associated with a low occurrence of oxidative damage in some tissues (Tøien et al. 2001; Orr et al. 2009). One method to quantify accrued oxidative damage is via telomere length measurement (Monaghan and Haussmann 2006; Monaghan 2010). Telomeres are the terminal sequences of eukaryotic chromosomes and can shorten over time due to incomplete DNA replication during cell division (Levy et al., 1992), chromosome end processing (Sfeir et al. 2005), and unrepaired, telomere-specific damage (von Zglinicki et al. 2000; von Zglinicki 2002). Telomere damage can occur when reactive oxygen species (ROS) released during cellular respiration cause single-strand breaks, which, left unrepaired, shorten the telomere over the next round of cell replication [von Zglinicki 2002; de Lange 2009; Shay and Wright 2019; see Reichert and Stier (2017) for a review on oxidative damage and telomere length *in vivo*].

Telomere dynamics in hibernators have been described in three species: Djungarian hamsters (*Phodopus sungorus*; Turbill et al. 2012), garden dormice (*Eliomys quercinus*; Giroud et al., 2014), and edible dormice (*Glis glis*; Turbill et al. 2013; Hoelzl et al. 2016). In these species, as in other small mammalian hibernators, most of hibernation is spent in torpor, which is characterized by low metabolic rates (as low as 2% of basal levels; Boyer and Barnes 1999), and T_b (Carey et al. 2003; Staples 2016). Since telomere shortening can be accelerated by oxidative damage (Kawanishi and Oikawa 2004), the depressed metabolism of torpor—which also stalls cell division (Kruman et al. 1988; Wu and Storey 2012)—is likely contributing to reported telomere length maintenance in *P. sungorus* (Turbill et al. 2012) and *G. glis* (Turbill et al. 2013). In contrast, any telomere shortening that does occur during hibernation has been correlated with the frequency of arousal episodes—the brief (~15 hour), metabolically-expensive returns to euthermic levels of T_b—that occur throughout hibernation (Giroud et al. 2014; Hoelzl et al. 2016).

All previous studies on telomere dynamics in hibernators have focused on animals that did not hibernate at subzero temperatures, and therefore did not need to maintain a thermal gradient between T_b and ambient temperature (T_a). In contrast, arctic ground squirrels (AGS; *Urocitellus parryii*) in northern Alaska are exposed to average winter soil temperatures of

-10°C, with a minimum of -26°C (Buck and Barnes 1999b). Under these conditions, they continuously use non-shivering thermogenesis during torpor bouts to defend a mean torpid core T_b of -1.7°C (Lee et al. 2015), and they can raise their metabolic rates by 36-fold during torpor to maintain a thermal gradient of up to 25°C between abdominal T_b and T_a (Richter et al. 2015). Furthermore, repeatedly arousing to euthermia (~37°C) under colder ambient conditions is energetically costly (Karpovich et al. 2009) and could induce higher levels of oxidative damage than arousing when exposed to milder conditions, which may ultimately shorten RTL.

Taken together, AGS hibernation physiology may impact telomeres differently than in hibernators adapted to milder climates, and we predicted that telomeres would show overall shortening across hibernation in AGS. We also investigated the effect of age to determine if telomere attrition during hibernation would translate to shorter telomeres with increasing age. Investigating telomere dynamics in additional hibernating species, particularly in those that exhibit dramatic fluctuations in physiological processes that impact telomere length, will provide a more holistic understanding of how telomere length changes in heterothermic mammals.

2.3 Materials and Methods

2.3.1 Study Species and Area

AGS are semi-fossorial rodents with a Holarctic distribution that includes Arctic Alaska (McLean 2018). Generally, females enter hibernation in August/early September and first emerge beginning in late April, while males hibernate for a shorter period, entering by early October and emerging in early April (Carl 1971; Sheriff et al. 2011). All AGS used in this study were captured adjacent to the Dalton Highway near the Atigun River (hereafter: Atigun; 68°27′N, 149°21′W; elevation 812 m) or near Toolik Lake (hereafter: Toolik; 68°38′N, 149°38′W; elevation 719 m) in northern Alaska, USA. Additional site characteristics are described in Sheriff et al. (2011).

2.3.2 Trapping and Age Designation

To collect tissue samples for telomere length measurement, we trapped and ear-tagged AGS over four time periods: mid-active season (7 to 14 July 2017), pre-hibernation (1 to 4 September 2017), post-emergence (20 to 25 April 2018), and a second mid-active season (29 June to 25 July 2018). To capture animals, we used Tomahawk Live Traps (14×14×40 cm; Tomahawk Live Trap Co.; Tomahawk, Wisconsin, USA) baited with carrot. Traps were generally

set out from 0900 to 1700, weather permitting (AGS are sensitive to unfavorable environmental conditions and may stay underground in inclement weather; Long et al. 2005; Williams et al. 2016).

In total, 21 animals, split into two groups, were sampled in this study. The first was an overwintering group (n=6), with each animal sampled in September 2017 (pre-hibernation) and again in April 2018 (post-emergence), which we used to compare telomere length before and after hibernation. The second was an age comparison group, sampled in either July 2017 (n=7) or July 2018 (n=8), with two animals sampled in both years, which we used to determine how age affects telomere length. For 20 of 21 animals, ages ranged from zero (born in summer captured) to three years old; the remaining animal was six years old. Any animal aged 1-3 used for this component of the study had been previously captured and tagged. Age was determined and designated as follows: upon initial capture, young-of-the-year animals (age zero) were identified from yearlings/adults by their relatively small mass and unmolted pelage. Because juveniles disperse in the fall, we assumed any unmarked adult AGS captured on the study site were yearlings that had dispersed the fall prior and thus designated them as one year old. At first capture, animals were given an ear tag (Monel No. 1005-1 tag; National Band and Tag Co.; Newport, Kentucky, USA) and a passive integrated transponder (PIT) tag (Avid Identification Systems, Inc; Norco, California, USA).

2.3.3 Ear Tissue Sampling

Trapped animals were transported to the Toolik Field Station for sampling. Animals were anesthetized via exposure to isoflurane vapors (Isothesia, Henry Schein; Dublin, OH, USA). Ear tissue was obtained with a 2 mm scissor punch (VWR; Radnor, Pennsylvania, USA) placed on the edge of the ear. The excised tissue was stored in 200 µl RNA*later* (Thermo Fisher Scientific; Waltham, Maine, USA), and frozen at -80°C for later DNA extraction. Animal trapping, housing, care, and sampling were carried out in accordance with approved Institutional Animal Care and Use Committee protocols (#1081763 and #340270) through the University of Alaska Fairbanks.

2.3.4 DNA Extraction and Relative Telomere Length (RTL) Measurement

Frozen ear samples were transported on dry ice to the University of Alaska Fairbanks. We extracted DNA from ear tissue using the QIAamp Fast DNA Tissue Kit (Qiagen; Hilden, Germany). Following extraction, DNA was stored in elution buffer (Qiagen) at -80°C for later

analysis. Prior to qPCR analyses, DNA extracts were purified using standard ethanol precipitation procedures. DNA concentration was determined with the Qubit 2.0 Fluorometer (Invitrogen; Carlsbad, CA, USA), and DNA quality was assessed with the NanoDrop One Spectrophotometer (Thermo Fisher Scientific). See Appendix for ethanol precipitation procedures and DNA concentrations and quality ratios.

We used qPCR to measure relative telomere length from ear tissue DNA according to methods described in Cawthon (2002). We determined relative telomere length (RTL) by measuring the factor by which an unknown sample differed from a standard sample in its ratio of telomere repeat copy number to non-variable gene copy number (non-VCN gene; Cawthon 2002; Equation 2.1). RTL is reflective of the average telomere length from the sampled tissue.

$$\mathrm{RTL} = \frac{E_C^{\,CqC} / E_T^{\,CqT}}{E_{SC}^{\,CqSC} / E_{ST}^{\,CqST}}$$

Equation 2.1. Equation for calculating relative telomere length. E=efficiency (expressed as 1 + *percent efficiency*; e.g. 98% efficiency is expressed as 1.98), Cq=quantification cycle, C=non-VCN sample, T=telomere sample, SC=non-VCN standard, ST=telomere standard.

All qPCR reactions were run on the 7900HT Fast Real-Time PCR System (Applied Biosystems; Foster City, California, USA). Glycogen synthase kinase-3 alpha (GSK3A) was used as the reference non-VCN gene (tested for non-variability in copy number after Smith et al., 2011). Primer sequences for the non-VCN gene were 5'-CTG ACA CTG CTG TCC TCA AG-3' (GSK3A-F) and 5'-CGA TGG ACG AGG TAT AAT CA-3' (GSK3A-R; Williams et al. 2011a). Telomeric primer sequences were 5'-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3' (tel1b) and 5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3' (tel2b; Epel et al. 2004). GSK3A and telomere qPCR assays were carried out in separate plates with 20 ng DNA, 400 nmol L^{-1} of each primer, 10 µl of Power SYBR Green Master Mix (Applied Biosystems), and 4.8 µl of molecular grade water (Thermo Fisher Scientific) per sample well.

A standard curve with a five-step serial dilution starting at 20 ng/µl DNA was also run on each plate. For the telomere assay, the efficiency was 98.4% and the mean coefficient of variation was 6.1%, while for the GSK3A assay the efficiency was 99.1% and the mean coefficient of variation was 4.1%. The thermal PCR profile for the GSK3A primers was 10 minutes at 95°C, followed by 40 cycles of 10 seconds at 95°C, 20 seconds at 59°C and 20 seconds at 72°C. For the telomere primers, the thermal profile was 10 minutes at 95°C, followed by 40 cycles of 10

seconds at 95°C, 20 seconds at 56°C and 20 seconds at 72°C. In each run, a final melting step was performed, ramping the temperature from 65 to 95°C in 1°C intervals. A no-template control (with molecular grade water) was included in each run. All samples and controls were run in triplicate. See Appendix for additional qPCR specifications.

2.3.5 Statistical Analyses

To determine the effect of hibernation on ear RTL, we used a paired t-test to compare the pre-hibernation and post-emergence RTL values for each of the six overwintering animals. The effect of age on ear RTL was tested with a linear mixed model, with "individual" as the random effect (two animals were sampled in both July 2017 and 2018). The six-year-old individual was omitted from the age model because of the sample size of one for that age group.

2.4 Results

Mean ear tissue RTL values did not change between sampling points that encompassed the hibernation season (paired t-test; t=-1.37, df=5, P=0.23). In five of the six animals, measures of post-hibernation RTL were longer compared to pre-hibernation RTL levels (Figure 2.1), which suggests that RTL in ear tissue is preserved across hibernation.

When we compared ear RTL from animals sampled in July, we did not find a significant effect of AGS age on RTL (linear mixed model; t=-1.69, df=3.33, P=0.18); inclusion of the one six-year-old animal in our analysis did not affect our results (t=-1.60, df=9.50, P=0.14). Variation in ear RTL within groups was relatively high (Figure 2.2): for one-year-olds, mean RTL±s.e.m. was 0.86±0.07, for two-year-olds 0.81±0.04, and for three-year-olds 0.78±0.04.

2.5 Discussion

We investigated if and how telomeres change length over the hibernation season and with age in a free-living arctic hibernator. We predicted that telomeres would shorten over the hibernation season, due to ROS exposure related to the high levels of thermogenesis during steady-state torpor and periodic arousal episodes. Instead, we found that telomeres were maintained across hibernation, which suggests that the overall low metabolism and suppression of cell division during hibernation is protective of telomeres. We found no effect of age on ear tissue RTL. However, due to our small sample size and high variation in RTL within age groups, additional studies are needed to ascertain whether telomere length varies with age in AGS.

In previous work on telomere dynamics in hibernators, torpor frequency (in animals that use daily torpor) and/or hibernation confers a protective effect on telomere length (Turbill et al. 2012; Turbill et al. 2013), and animals that arouse more often or spend more time euthermic during hibernation experience greater telomere attrition (Giroud et al. 2014; Hoelzl et al. 2016). In contrast, AGS in the northern part of their range become thermogenic during torpor when hibernacula temperatures drop below the freezing points of their tissues, and this heightened metabolic rate may produce ROS that could subsequently shorten telomeres. Contrary to our predictions, we found that telomere length is maintained throughout torpor in AGS, a result similar to those found in studies of temperate hibernators (Turbill et al. 2012; Turbill et al. 2013).

When considering why telomeres are maintained throughout hibernation in AGS, it is necessary to consider the potential effects of both thermogenic torpor (Buck and Barnes 2000) and energetically-expensive arousals (Karpovich et al. 2009) when hibernating at subzero T_a. In terms of the effect of thermogenic torpor bouts, the necessary increase in metabolic rates to support thermogenesis over torpor in AGS may not be sufficient to induce ROS-mediated telomere shortening, particularly in peripheral tissues like the ear. Although metabolic rate during torpor at an ambient temperature of -16°C is 18-fold higher when compared to torpid metabolic rate at 0°C (Buck and Barnes 2000), this is still only ~32% of basal levels (Richter et al. 2015). Arousing at subzero T_a is also more energetically expensive than arousing at milder T_a: Karpovich et al. (2009) found that average metabolic rate during interbout euthermia was ~47% higher in AGS housed at -12°C compared to 2°C, which is ~176% of basal rates. In spite of this dramatic arousal physiology, which would presumably shorten telomeres via resumed cell division and ROS release, telomere length was maintained in AGS.

Alternatively, it is possible that the increase in metabolic rates (from both torpor and arousals) at low soil temperatures did cause telomere shortening, but these effects were counteracted by telomere lengthening via telomerase, the enzyme that adds lost telomeric repeats (reviewed in Shay and Wright 2019) and is active in rodent somatic tissues (Prowse and Greider 1995; Seluanov et al. 2007; Gorbunova and Seluanov 2009). As in other rodents, telomerase might be active in somatic tissues of AGS and this could potentially reverse telomere shortening caused by increased metabolic rate or oxidative damage over arousals (Tøien et al. 2001; Orr et al. 2009). To determine if telomerase is active during hibernation in bats, Wang et al. (2011) compared telomerase activity between two species and found that the hibernating

species exhibited higher levels of telomerase activity compared to the non-hibernating species in all tissues measured. To date, this is the only study that has measured telomerase activity in a hibernator; clearly more work is needed to understand how this enzyme might contribute to hibernator telomere dynamics. In moving forward, and if telomerase is indeed active in AGS somatic tissues, it will be also pertinent to consider how this enzyme might influence male telomere dynamics during periods of underground euthermia that can occur before or after heterothermy has ceased (Barnes 1996; Williams et al. 2011b).

Past studies in temperate hibernators used peripheral tissues to determine telomere length change over hibernation: Turbill et al. (2012) and (2013) used ear tissue from *P. sungorus* and *G. glis*, respectively, while Giroud et al. (2014) and Hoelzl et al. (2016) used buccal cells from *E. quercinus* and *G. glis*, respectively. The current study also capitalized on both the ease of sampling and minimal harm to the animal by using a peripheral tissue to understand telomere dynamics in AGS. However, tissue dynamics differ between somatic tissues with varying thermogenic demands in zebra finches (Reichert et al. 2013), and Wilbur et al. (Chapter 3 of this thesis) found that telomere shortening varied across tissues in captive AGS hibernating at 2°C. Specifically, while telomere length in liver and heart was similar in animals sampled at mid- vs. late hibernation, telomeres were shorter in brown adipose tissue at late hibernation. This difference may relate to the thermogenic role of brown adipose tissue during hibernation (Cannon and Nedergaard 2004; Ballinger and Andrews 2018), given that ROS are released in this tissue upon uncoupling protein 1 activation in mice (Chouchani et al. 2016). A complete understanding of how telomeres change in hibernating mammals might best be approached by measuring telomere dynamics in multiple somatic tissues, including peripheral tissues that are easily sampled and more metabolically-active internal organs.

We predicted that any observed telomere length change throughout hibernation would translate into telomere length change with increasing age. Although we cannot say for certain how increasing age impacts telomere length in AGS [at least in ear tissue; see Chapter 3 of this thesis for differences in telomere lengths between brown adipose tissue (BAT), liver, and heart in juvenile and adult AGS], it is clear from our results that there is high variability in ear RTL, indicating that larger sample sizes are essential for determining patterns when using a cross-sectional approach as we have done here. Long-term longitudinal sampling may reduce the complication of between-individual variation in RTL and could reveal any effect of age cohort on telomere length (Hall et al. 2004). Future work should seek a balanced sex ratio in sampling and

measure at different time points across the active season to further pinpoint how telomeres shorten with age in AGS.

Despite the high metabolic rates required to both defend T_b against subfreezing T_a during torpor and to reach and maintain euthermic T_b that may be ~60°C above T_a, telomeres were maintained across hibernation in AGS. Although we can't be certain of the mechanism behind this molecular preservation, it suggests that AGS maintain cellular integrity in the face of extreme environmental conditions. A future area of work should include biopsies from tissues that are more directly implicated in the arousal process, including brown adipose tissue, liver, and heart, to complement recent work by Wilbur et al. (unpublished report); it would be intriguing to measure telomere dynamics in these other tissues in a free-living population exposed to subfreezing T_a.

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2.8 Figures

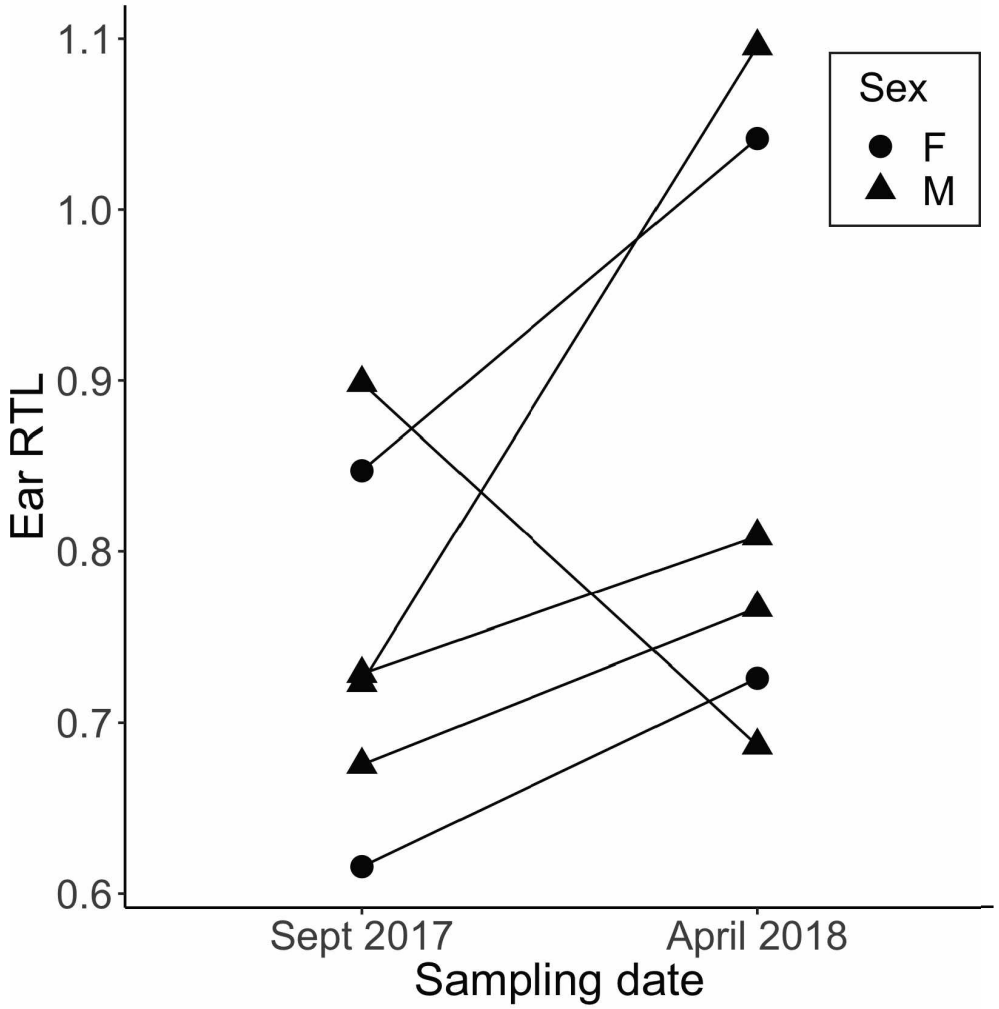

Figure 2.1. Telomere maintenance across hibernation. Differences in telomere length in six AGS between September 2017 (pre-hibernation) to April 2018 (post-hibernation). Although all but one individual lengthened their telomeres over hibernation, average values did not significantly change (P=0.23). Removal of the individual that showed telomere shortening produced a significant effect (P=0.04).

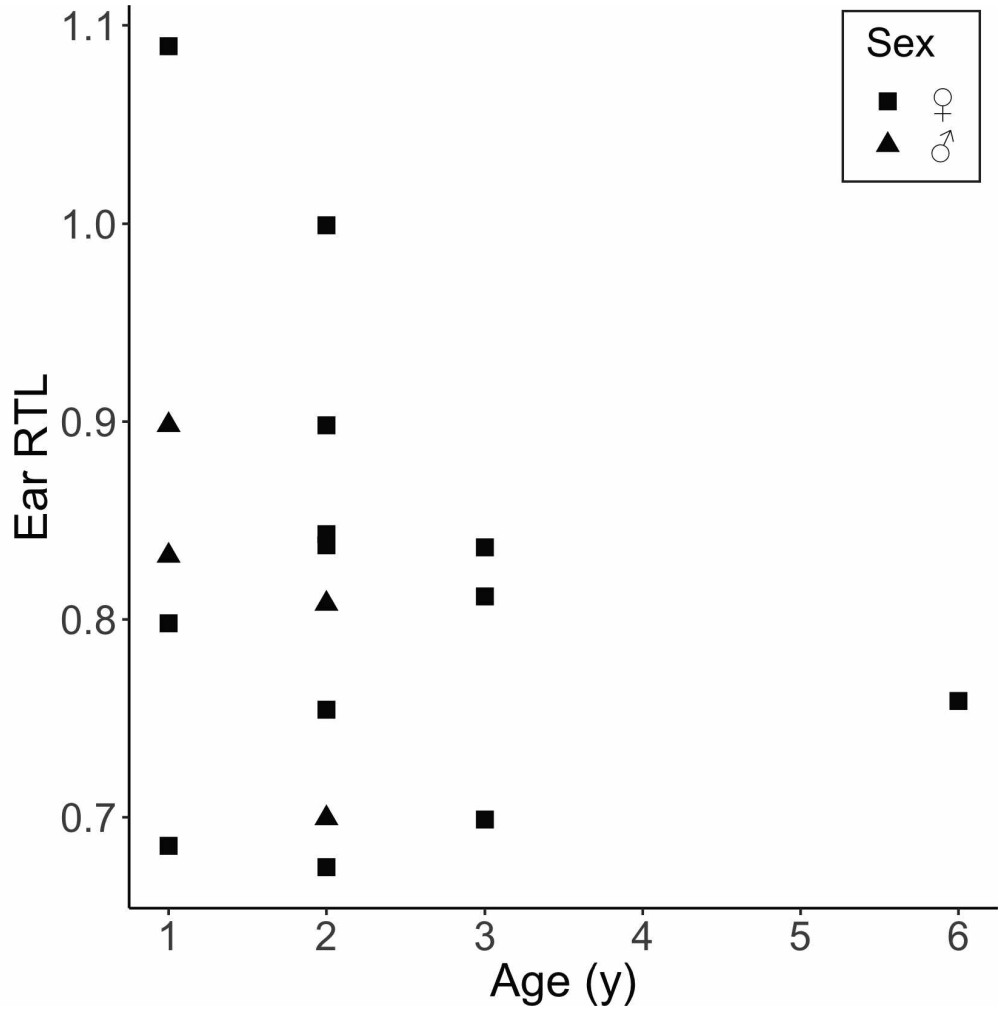

Figure 2.2. Effect of age on telomere length. RTL trended downward with age, but this effect was not significant (n=14; *P*=0.18). The single six-year-old was not included in our analysis but is retained here for visual comparison between ages.

Chapter 3: Tissue-Specific Telomere Dynamics in Hibernating Arctic Ground Squirrels[3]

3.1 Abstract

Hibernation is used by a variety of mammals to survive seasonal periods of resource scarcity. However, reactive oxygen species (ROS) released during periodic rewarming induce oxidative damage in some tissues. Telomeres, which are the dynamic terminal sequences of chromosomes, may shorten in the presence of ROS, and thus the telomere length of an individual reflects the degree of accrued oxidative damage. This study quantified telomere length dynamics throughout hibernation in arctic ground squirrels (*Urocitellus parryii*). We hypothesized that telomere dynamics are tissue-specific and predicted that telomere shortening would be most pronounced in brown adipose tissue (BAT), the organ that directly supports non-shivering thermogenesis during periodic arousals via mitochondrial uncoupling. DNA was extracted from liver, heart, and BAT of 46 juvenile and adult animals sampled either at the middle or end of hibernation. We used qPCR to determine relative telomere length (RTL) for each tissue. Age did not have a significant effect on RTL in any tissue. In juvenile females, RTL in BAT—but not in liver and heart—was shorter at late hibernation than at mid-hibernation. Additionally, mid-hibernation BAT RTL was longer than in liver and heart. At late hibernation, juvenile males had longer RTL in BAT than juvenile females, perhaps due to differences in hibernation duration between the sexes. Finally, BAT RTL at late hibernation negatively correlated with arousal frequencies across hibernation. Overall, we show that telomere shortening occurs in a tissue-specific manner in a hibernating mammal.

[3] Wilbur, S.M., Barnes, B.M., Kitaysky, A.S., Williams, C.T. Tissue-specific telomere dynamics in hibernating arctic ground squirrels (*Urocitellus parryii*). In review at *Journal of Experimental Biology*.

3.2 Introduction

Hibernation is used by diverse species of mammals and one bird species to survive seasonal periods of food scarcity (Geiser, 1998; Staples, 2016). Hibernation is associated with longer maximum lifespans than predicted by body mass, a phenomenon that is supported by both predator avoidance and an overall slower pace of living (Turbill et al., 2011). If extrinsic mortality is diminished—as is the case when an animal is hibernating and protected from predation—selection should favor somatic maintenance (Kirkwood and Austad, 2000). One way to quantify the degree of incurred somatic damage and/or maintenance is via telomere length measurement (Monaghan and Haussmann, 2006; Monaghan, 2010). Telomeres are repetitive ($TTAGGG_n$ in vertebrates), nucleoprotein structures at the ends of linear chromosomes (Blackburn, 1991; Blackburn et al., 2015). These complex and highly-regulated sequences protect genomic DNA by preventing chromosome end-to-end fusion and by buffering interstitial DNA from the "end-replication problem," whereby the lagging strand loses terminal nucleotide bases with each cell division (Levy et al., 1992; de Lange, 2009; Greider, 2016). Telomeres can shorten as organisms age due to cell replication and in response to oxidative damage (von Zglinicki, 2002), and critically short telomeres can accelerate cellular aging by triggering cell senescence pathways (Collado et al., 2007). In terms of its ability to predict longevity or diagnose disease, telomere length is perhaps best viewed as a contributing, rather than a direct, measure of overall organismal health (Blackburn et al., 2015).

Hibernation in small mammals is characterized by two dramatically different physiological states: prolonged, multi-day torpor (metabolism depressed below basal rates and low body temperature) and brief (<1 day) intermittent arousals (rapid rises in metabolic activity and body temperature; Carey et al., 2003; Ruf and Geiser, 2015). During torpor, average heart rate across hibernators slows from 155 to 9 beats min^{-1} (Zatzman, 1984), core body temperature can drop as low as -2.9°C (Barnes, 1989), and cell division is arrested (Kruman et al., 1988; Wu and Storey, 2012). To fuel rewarming during periodic arousals from torpor, small mammalian hibernators increase their oxygen consumption by 300-fold over minimum hibernation levels (Karpovich et al., 2009). This dramatic surge in oxygen uptake, coupled with a pronounced increase in metabolic demand, can cause tissue-specific oxidative damage over an arousal episode (Carey et al., 2000; Orr et al., 2009). An organ that directly supports thermogenesis during arousal episodes is brown adipose tissue (BAT; Cannon and Nedergaard, 2004). In mice, heat production associated with uncoupling protein 1 (UCP1) activation in BAT mitochondria is accompanied by elevated levels of highly unstable reactive oxygen species (ROS; Chouchani et

al., 2016), molecules that can interfere with and damage lipids, proteins, and DNA. ROS produced in BAT mitochondria may damage telomeres by inducing DNA lesions, which can dramatically shorten telomeres over the subsequent cell cycle (von Zglinicki et al., 2000) and ultimately induce cell senescence (von Zglinicki et al., 2005). Although essentially all organs become more metabolically active over arousal episodes, BAT is the only organ to possess ROS-generating UCP1 (Rousset et al., 2004) and therefore has relatively higher potential to experience telomere shortening via oxidative damage.

The use of telomere length as a biological marker for cellular or organismal aging in hibernators has been investigated in three species: Djungarian hamsters (*Phodopus sungorus*; Turbill et al., 2012), garden dormice (*Eliomys quercinus*; Giroud et al., 2014), and edible dormice (*Glis glis*; Turbill et al., 2013; Hoelzl et al., 2016). In general, this work demonstrated that the use of torpor preserves telomere length, while arousal frequency and/or the number of arousals experienced throughout hibernation are/is positively correlated with the degree of telomere shortening. In these studies, all measures of telomere length were from peripheral tissues (ear or buccal cells). While these tissues allow for non-invasive and repeat sampling, ear tissue and cheek cells likely experience little cell turnover or ROS-mediated oxidative damage throughout hibernation and may not be reflective of telomere dynamics in other tissues. Therefore, it is worth investigating hibernator telomere dynamics in additional tissues—including those that experience a surge in ROS release over arousal episodes—to determine if telomere length change is a local or systemic phenomenon.

In this study, we quantified relative telomere length (RTL) dynamics in three tissues (BAT, liver, and heart) in hibernating arctic ground squirrels (AGS; *Urocitellus parryii*). Liver and heart are appropriate comparison tissues to BAT in determining how ROS production might affect RTL. Unlike BAT, liver and heart do not have UCP1 in their mitochondria. Additionally, liver does not experience ROS-mediated oxidative damage during hibernation (Orr et al., 2009; Brown et al., 2012) and ROS production in heart during late torpor is counteracted by antioxidants in the subsequent arousal episode (Wei et al., 2018). To test if RTL shortened throughout hibernation, we sampled juvenile females at the middle (early January) and end (mid-March) of their hibernation season. As age and sex can affect telomere length dynamics (Monaghan, 2010), we also examined whether RTL differed among tissues across age and sex cohorts sampled late in hibernation. We hypothesized that hibernator RTL dynamics are tissue-

specific and predicted that RTL in BAT would exhibit the most pronounced shortening of the three tissues due to elevated ROS exposure during arousal episodes.

3.3 Materials and Methods

3.3.1 Ethics Statement

All animal trapping, housing, care, and sampling were carried out in accordance with approved IACUC protocols (#1081763 and #340270) through the University of Alaska Fairbanks and with state (ADF&G #17-100) and federal (BLM #F-94817) permits.

3.3.2 Animals

AGS are semi-fossorial rodents with a Holarctic distribution that includes Arctic Alaska (McLean, 2018). Maximum recorded ages for AGS from our study region are ten years for females and six years for males (unpublished data). All AGS used in this study were captured adjacent to the Dalton Highway near the Atigun River in northern Alaska (68°27′N, 149°21′W) in July 2017 and transported by truck to the University of Alaska Fairbanks. Prior to initiating hibernation, animals were housed in 48×32×32 cm hanging metal cages and provided cotton bedding for nest construction. Initial ambient conditions were 20°C and 12L:12D photoperiod; 10 pellets per day of rodent chow (Mazuri; St. Louis, MO, USA) and water *ad libitum* were provided throughout the active, pre-hibernation period.

Beginning August 1, animals were gradually transitioned (loss of 30 minutes of light per day) to a short photoperiod (4L:20D) to mimic arctic day lengths in autumn. Upon detection of hibernation readiness (e.g. not eating, quiet, curled up in nest) and before 1 December, animals were moved to environmental chambers with an ambient temperature of 2°C and a 0L:24D photoperiod. Torpid animals were transferred into 43×27×19 cm plastic tubs (Nalgene; Rochester, NY, USA), food was withheld, and gel packs (HydroGel; Portland, ME, USA) were provided for access to water. During hibernation, we monitored the animals by opening their cotton bedding and placing wood shavings on the animals' exposed backs. We inspected daily to assess—by the presence or absence of shavings—the duration of torpor bouts and occurrence of arousal episodes (Pengelley and Fisher, 1961).

We used three groups of animals in this study: juvenile females (n=21), adult (>1 year) females (n=10), and juvenile males (n=14). The two latter groups were from a concurrent experiment and were included opportunistically to augment late hibernation samplings. To

represent mid-hibernation (MH), 11 juvenile females were randomly selected and sampled in early January 2017. For late hibernation (LH), the remaining 10 juvenile females were sampled in mid-March 2017. Some adult females (n=7) and juvenile males (n=6) were sampled at LH, while the remainder were sampled three (n=2), eight (n=3), or fifteen (n=6) days after animals spontaneously ended hibernation (for sampling date details, see Table A2). For post-hibernation adult females and juvenile males, 10 pellets of rodent chow per day were provided from three days post-emergence to the day of sampling. Note: LH and post-hibernation adult females and juvenile males will hereafter be collectively designated as LH animals, as no significant differences in RTL were found between LH and post-hibernation in either age-sex group.

3.3.3 Tissue Sampling

Approximately 18 hours before sampling, we induced animals to begin arousing from torpor via 10-15 minutes of gentle hand manipulation before returning them to their nests. Immediately prior to sampling, aroused animals (core temperature >30°C) were anesthetized via exposure to isoflurane vapors (Isothesia, Henry Schein; Dublin, OH, USA). Animals were euthanized by decapitation before we excised approximately 1.5 g each of liver, whole heart, and intrascapular BAT. The samples were then placed on RNase AWAY-treated foil (Thermo Fisher Scientific; Waltham, MA, USA), flash-frozen in liquid nitrogen, and stored at -80°C for later DNA extraction.

3.3.4 DNA Extraction

We extracted DNA from liver, heart, and BAT using the QIAamp Fast DNA Tissue Kit (Qiagen; Hilden, Germany). Following extraction, samples were stored in TAE buffer (Qiagen) at -80°C for later analysis. Before running qPCR, all extracts were purified using standard ethanol precipitation procedures, as follows: 50 µl of extracted DNA was combined with 150 µl of absolute ethanol, 5 µl of sodium acetate (3M, pH 5.2), and 1 µl of glycogen (all reagents from Thermo Fisher Scientific) and allowed to incubate overnight (at least 15 hours) at -20°C. Samples were centrifuged for 30 minutes at 13,400 rpm. After this initial spin, the ethanol mixture was poured off, 500 µl of 75% ethanol was added, and the samples were spun at 13,400 rpm for 10 minutes; this wash step was repeated twice. After the final ethanol removal, samples were allowed to air dry for 10 minutes until the pellet was completely dry. 50 µl TAE buffer was added to resuspend the DNA pellet.

DNA concentration was determined with the Qubit 2.0 Fluorometer (Invitrogen; Carlsbad, CA, USA), and DNA quality was assessed with the NanoDrop One Spectrophotometer (Thermo Fisher Scientific). All samples used in this study contained at least 14.6 ng μl^{-1} DNA with quality ratios between 1.78-2.08 for A260/A280 (a measure of protein contamination) and 1.72-2.83 for A260/A230 (a measure of phenol and/or salt contamination).

3.3.5 Telomere Length Assessment

Quantitative polymerase chain reaction (qPCR) was used to quantify tissue-specific telomere length in hibernating AGS. RTL was determined by measuring the factor by which an unknown sample differed from a standard sample in its ratio of telomere repeat copy number to non-variable gene copy number (non-VCN gene; Equation 3.1). RTL is reflective of the average telomere length from the sampled tissue (Cawthon, 2002).

$$\mathrm{RTL} = \frac{E_C^{\,CqC} / E_T^{\,CqT}}{E_{SC}^{\,CqSC} / E_{ST}^{\,CqST}}$$

Equation 3.1. Equation for calculating relative telomere length (RTL; Cawthon, 2002). E=primer efficiency (expressed as $1 + percent\ efficiency$; e.g. 98% efficiency is expressed as 1.98), Cq=quantification cycle, C=non-VCN sample, T=telomere sample, SC=non-VCN standard, ST=telomere standard.

All qPCR reactions were run on the 7900HT Fast Real-Time PCR System (Applied Biosystems; Foster City, CA, USA). Glycogen synthase kinase-3 alpha (GSK3A) was used as the reference non-VCN gene (tested for non-variability in copy number after Smith et al., 2011). Primer sequences for the non-VCN gene were 5'-CTG ACA CTG CTG TCC TCA AG-3' (GSK3A-F) and 5'-CGA TGG ACG AGG TAT AAT CA-3' (GSK3A-R; Williams et al., 2011). Telomeric primer sequences were 5'-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3' (tel1b) and 5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3' (tel2b; Epel et al., 2004). GSK3A and telomere qPCR assays were carried out in separate plates with 20 ng DNA, 400 nmol L^{-1} of each primer, 10 µl of Power SYBR Green Master Mix (Applied Biosystems), and 4.8 µl of molecular grade water (Thermo Fisher Scientific) per sample well. A standard curve with a five-step serial dilution starting at 20 ng μl^{-1} DNA was also run on each plate. Using the equation $Efficiency = -1 + 10^{-\frac{1}{slope}}$, the slope of each run's standard curve was used to calculate primer efficiencies for each plate.

The thermal PCR profile for the GSK3A primers was 10 minutes at 95°C, followed by 40 cycles of 10 seconds at 95°C, 20 seconds at 59°C, and 20 seconds at 72°C. For the telomere primers, the thermal profile was 10 minutes at 95°C, followed by 40 cycles of 10 seconds at 95°C, 20 seconds at 56°C, and 20 seconds at 72°C. In each run, a final melting step was performed, ramping the temperature from 65 to 95°C in 1°C intervals. A no-template control (with molecular grade water) was included in each run. All samples and controls were run in triplicate. Hard-shell 384-well PCR plates (thin wall, skirted, clear; Bio-Rad; Hercules, CA, USA) and MicroAmp Optical Adhesive Film (Applied Biosystems) were used for all runs. See Appendix for primer efficiencies and coefficients of variation per plate (Table A3).

3.3.6 Statistical Analyses

All analyses were performed in R (R Core Team, 2018). Averages are reported as mean±s.e.m. We ran linear mixed models to test the following: 1) the effects of hibernation stage, tissue, and the interaction between stage and tissue on RTL within juvenile females, 2) the effects of age, tissue, and the interaction between age and tissue on RTL within LH females, and 3) the effects of sex, tissue, and the interaction between sex and tissue on RTL within LH juveniles. In the case of a significant interaction, planned pairwise comparisons were run within each factor of the interaction. We used the Kenward-Rogers method to determine degrees of freedom, and used the Tukey method for multiple comparisons to adjust *p*-values. Based on our finding that BAT RTL differed between MH and LH, we subsequently tested the effect of arousal frequency on BAT RTL in LH animals using a linear model; age and sex were included as additional factors. Finally, we used Pearson's correlation tests to determine whether RTL was correlated across tissues. We performed these correlation analyses via two approaches: 1) by including all animals and 2) within a group (e.g. MH juvenile females).

3.4 Results

3.4.1 Hibernation in AGS

Hibernation durations ranged from 79 to 196 days. Juvenile females were sampled 90.5±1.6 days (MH group) and 166.5±1.7 days (LH group) after initiating hibernation. Adult females were sampled after 168.5±4.5 days of hibernation and juvenile males after 149.9±3.0 days. The average number of arousals throughout hibernation per group were as follows: 6.5±0.2 (MH juvenile females), 11.5±0.2 (LH juvenile females), 11.4±0.4 (adult females), 10.3±0.2 (juvenile males). Generally, animals aroused at similar frequencies (per month):

2.1±0.1 (MH juvenile females), 2.1±0.02 (LH juvenile females), 2.0±0.05 (adult females), and 2.0±0.03 (juvenile males). AGS spent 91.8±0.04% of the hibernation period in torpor.

3.4.2 Effects of Stage, Age, and Sex on RTL

We quantified RTL dynamics in three tissues from three groups of AGS: juvenile females at both MH and LH (stage effect), adult females at LH (age effect), and juvenile males at LH (sex effect). In juvenile females, RTL in BAT was shorter at LH than at MH ($P<0.001$; Figure 3.1). Additionally, BAT RTL was longer than heart ($P<0.001$) and liver ($P<0.001$) RTL at MH. There was no difference in heart RTL ($P=0.58$) or liver RTL ($P=0.19$) RTL between hibernation stages (Figure 3.1). In LH females, there was no significant interaction between tissue and age ($P=0.17$; Figure 3.2). Juvenile males sampled at LH had longer RTL in BAT than in heart ($P<0.001$) and in liver ($P<0.001$; Figure 3.3). Finally, at LH, BAT RTL in juvenile males was longer than BAT RTL in juvenile females ($P=0.01$; Figure 3.3).

3.4.3 Effect of Arousal Frequency on BAT RTL

We found a significantly negative relationship between average monthly arousal frequency and BAT RTL ($P=0.001$ via linear model; n=34; Figure 3.4) in LH animals. Additional factors age ($P=0.45$) and sex ($P=0.08$) did not impact BAT RTL. See Table A4 for correlations between hibernation parameters.

3.4.4 RTL correlation between tissues

When including all animals, we found no significant correlations in RTL between tissues (Table 3.1 and Figure 3.5). Within groups, liver RTL correlated with BAT RTL in LH adult females, but this association was negative (r=-0.74; $P=0.01$). In all other groups, there were no significant correlations between tissues.

3.5 Discussion

Although hibernation is an effective and widely used survival strategy in mammals, it comes at a cost: hibernators experience ROS-mediated oxidative damage in some tissues over arousal episodes (Carey et al., 2000; Orr et al., 2009), which may be reflected in an individual's telomere length. To complement past studies that have explored the effects of hibernation on RTL in a single peripheral tissue, we thought it relevant to measure RTL in multiple internal tissues, particularly in BAT, the organ that fuels non-shivering thermogenesis over arousal episodes. We found compelling evidence for tissue-specific RTL shortening in hibernating AGS.

In particular, BAT RTL in juvenile females was dramatically shorter at LH than at MH and BAT RTL was significantly longer than RTL in liver and heart at MH. In contrast, liver and heart RTL were very similar between MH and LH in juvenile females, further highlighting the tissue-specific nature of RTL shortening in AGS.

While this study focused on RTL shortening as a product of metabolic activity and ROS-mediated oxidative damage, initial telomere research was driven by their potential usefulness as biomarkers for aging rates and longevity (e.g. Harley et al., 1990; Harley et al., 1992; Rudolph et al., 1999; López-Otín et al., 2013). More recently, there has been an interest in quantifying telomere dynamics in non-model organisms, including hibernating mammals (e.g. Hoelzl et al., 2016). These efforts have revealed the great biodiversity of telomere dynamics across organisms and have expanded our understanding of telomere biology beyond its implications for human health. While it has been shown that telomere dynamics can be specific to age, sex, and species, universal mechanisms have also emerged from this body of work, including the deleterious effect of oxidative damage and the tissue-specific nature of telomere length change.

All previous studies that have sought to find a relationship between telomere length and hibernation have connected telomere dynamics with torpor use or arousal frequency, as these two physiological states differentially influence ROS production (Orr et al., 2009). Initial work suggested that torpor use confers a protective effect on RTL, with demonstrations of RTL stasis or even lengthening across the hibernation season in Djungarian hamsters (*P. sungorus*; Turbill et al., 2012) and edible dormice (*G. glis*; Turbill et al., 2013). Later work found evidence for telomere shortening as a product of arousal frequency and time spent euthermic. In garden dormice (*E. quercinus*) that displayed short arousal episodes during the first month hibernating, buccal cell RTL did not significantly change over the hibernation season. However, in individuals with long arousal episodes in the first month of hibernation, overwinter changes in RTL were negatively associated with time spent euthermic (Giroud et al., 2014). Hoelzl et al. (2016) found a similar relationship between euthermia and RTL shortening: RTL in *G. glis* buccal cells significantly shortened over the hibernation season and the best predictors of this effect were arousal number and arousal frequency.

The current study adds an important component to prior work on telomere dynamics in hibernating mammals and adds additional support for the relationship between arousal frequency and telomere shortening. In our study, juvenile females sampled at LH had

significantly shorter RTL in BAT than at MH, likely due to the greater number of arousals experienced by the LH animals. BAT, the organ responsible for thermogenesis at the initiation of an arousal episode, is highly metabolically active during these periods and experiences arousal-induced oxidative damage (Orr et al., 2009). This damage is likely due to the large quantities of mitochondrial ROS released in BAT upon thermogenic activation; the level of ROS released is presumably above the amount found in other active tissues (Chouchani et al., 2016). [ROS appear to be essential signaling molecules in supporting thermogenesis, to the degree that pharmacological depletion of mitochondrial ROS in BAT results in hypothermia upon cold exposure (Chouchani et al., 2016)]. In our study, not only was BAT RTL shorter at LH than at MH, but RTL in this tissue was much greater than in liver or in heart at MH. This finding is particularly intriguing in that it suggests that AGS "prepare" for significant and predictable hibernation-induced shortening in this tissue only.

The dramatic difference in BAT RTL between stages is made more significant by the fact that RTL did not appreciably change in liver and heart tissues, neither of which are expected to experience UCP1-induced ROS release as these tissues lack this protein in their mitochondria. In addition, Orr et al. (2009) found that oxidative stress markers in liver did not differ between torpid and aroused AGS, and that oxidative stress was not associated with torpor in several tissues (including liver). In heart tissue, ROS that accumulate during torpor are counteracted by antioxidants released during the subsequent arousal (Wei et al., 2018). Thus, previous work suggests that neither liver nor heart experience a significant, enduring ROS load that might shorten telomeres. There is an additional possibility that telomerase—the enzyme that lengthens telomeres—could be active in liver and heart throughout hibernation. While telomerase activity in most human somatic cells is low (Cong et al., 2002), in somatic cells of smaller mammals (particularly rodents) the enzyme is comparatively more active, and its activity varies in a tissue-specific manner (Prowse and Greider, 1995; Seluanov et al., 2007; Gorbunova and Seluanov, 2009). To date, there has been one published study that directly measured telomerase activity in a hibernator: Wang et al. (2011) detected telomerase activity in two bat species (*Hipposideros armiger* and *Rousettus leschenaultia*) that differed in their use of hibernation. In both species, telomerase activity was higher in metabolically active tissues (liver, spleen, and kidney). In the heterothermic species (*H. armiger*), telomerase activity was higher than in *R. leschenaultia*, and this difference was even more pronounced when *H. armiger* was hibernating (Wang et al., 2011). Although further studies are needed, this previous work in bats supports the idea that telomerase could be impacting telomere length in other hibernating species.

DNA repair throughout hibernation should also be considered for its potential impact on telomere length. ROS interact with the guanine bases of telomeres to produce 8-oxo-7,8-dihydroguanine, a lesion that can be removed and repaired via base excision repair pathways (Rhee et al., 2010; Wang et al., 2010; Fouquerel et al., 2013). Although studies on DNA repair in hibernators are very limited, it appears that DNA repair mechanisms are shut down during torpor (or upon hypothermia induction; e.g. Baird et al., 2011) and resumed upon arousal (Yancey et al., 2018). This pattern of repair shutdown agrees with the slowing or cessation of many other physiological and molecular processes during torpor and their resumption upon the next arousal cycle (e.g. cell division, mitosis, transcription/translation; reviewed in Carey et al., 2003). One exception to this rule was noted in Schwartz et al. (2013): DNA repair genes such as *RAD50* are elevated during torpor in the hypothalamus of thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*). However, repair dynamics in one tissue do not necessarily imply similar dynamics in any other, and it remains to be seen how DNA repair mechanisms operate in other tissues across hibernation. In our study, perhaps repair of telomere lesions is occurring in AGS liver and heart over arousals, which would support the lack of RTL change we saw in these tissues, but any potential repair activity in BAT may be overwhelmed by the presumed release of ROS in this tissue alone.

In addition to the effect of hibernation stage on AGS RTL, we also considered the effect of age. In adult and juvenile females sampled at LH, we found no differences in tissue-specific RTL. This was surprising, as we anticipated older females would have shorter RTL in BAT due to ROS exposure across multiple hibernation seasons. This apparent preservation of telomere length could be due to active season telomerase activity: perhaps telomeres shortened over hibernation are restored by telomerase during the subsequent summer, thus preparing BAT telomeres for another round of hibernation-induced shortening. We also included juvenile males in our study to investigate a potential sex effect on RTL. Juvenile males had significantly longer RTL in BAT than in liver and heart at LH and longer BAT RTL than seen in juvenile females. This pattern was likely due to differences in hibernation duration between these two groups: females hibernated longer than males, a pattern also seen in free-living populations (Sheriff et al., 2011). Presumably, if males and females had hibernated for equal duration, we would not see tissue-specific differences in RTL between the sexes; overall, we cannot say with certainty whether rates of RTL shortening differ between the sexes.

Our finding that arousal frequency negatively correlates with RTL in BAT—in other words, the more frequently an animal aroused the shorter were its BAT telomeres—is important because it suggests that the arousal episode is a driving force for telomere shortening in BAT. This effect is made further significant in that it agrees with past hibernator-telomere work that also found a negative effect of arousal frequency on RTL in dormice buccal cells (Giroud et al., 2014; Hoelzl et al., 2016). Finally, we found no significant positive correlations between RTL across tissues. This is likely explained by tissue-specific differences in both the degree of accumulated oxidative damage at telomeres and by potential telomerase activity. Considering how hibernator tissues differ in their metabolic contribution to periodic rewarming, future investigations should take care in selecting tissues with the understanding that telomere dynamics in one do not necessarily represent those in another.

This study is the first to quantify telomere dynamics from multiple, internal tissues in a hibernating animal—including BAT, the organ that fuels non-shivering thermogenesis at arousal initiation—and expands previous hibernator-telomere work to include a ground squirrel species that is adapted to extreme conditions and seasonality. Future studies should include measures of telomerase activity (sampling during torpor, over arousal episodes, and over the active seasons); additionally, obtaining direct measures of ROS would provide a more holistic picture of what is driving telomere length dynamics throughout hibernation. Considering the possibility of tissue biopsies, a longitudinal study of telomere dynamics (starting with animals at pre-hibernation and continuing throughout the season) in BAT would be useful in understanding precisely how telomere length changes in BAT within an individual.

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3.8 Figures

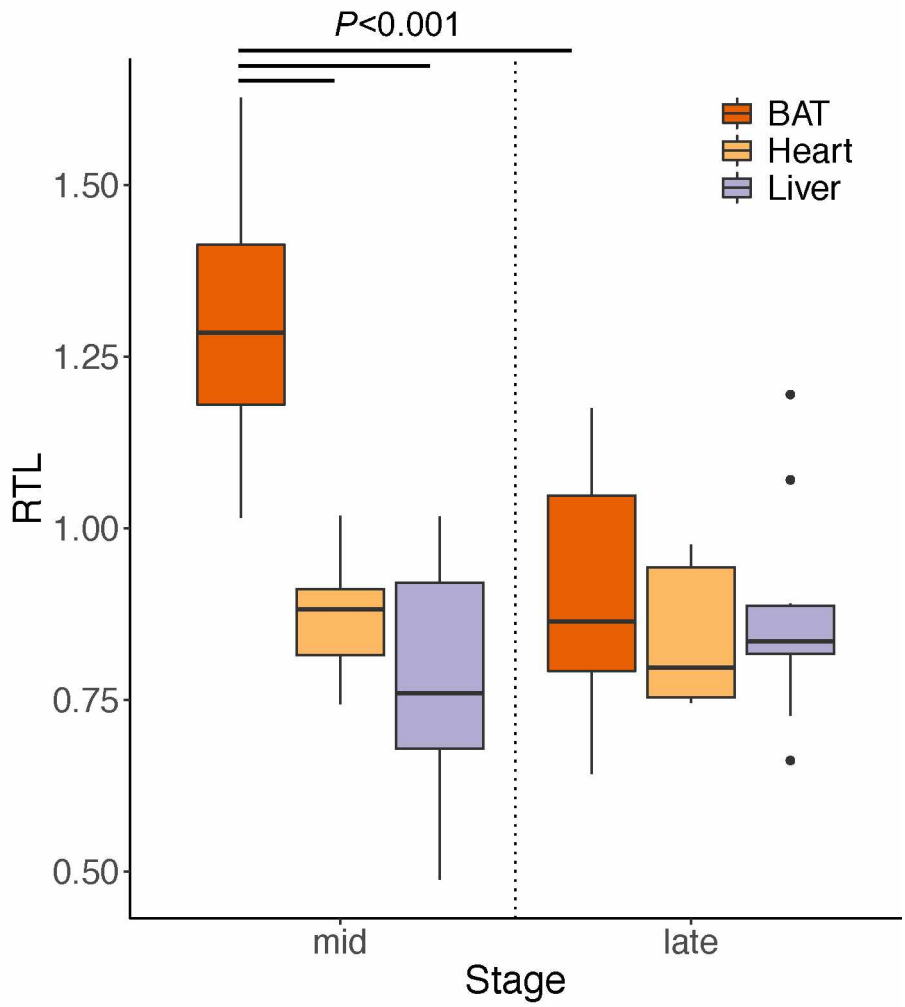

Figure 3.1. Relative telomere length (RTL) in juvenile females at mid- and late hibernation. At mid-hibernation, brown adipose tissue (BAT) RTL was greater than heart ($P<0.001$ via linear mixed model) and liver ($P<0.001$). Mid-hibernation BAT RTL was also greater than at late hibernation ($P<0.001$). 11 juvenile females were sampled at mid-hibernation and 10 at late hibernation. Bars indicate significant differences between groups within planned comparisons.

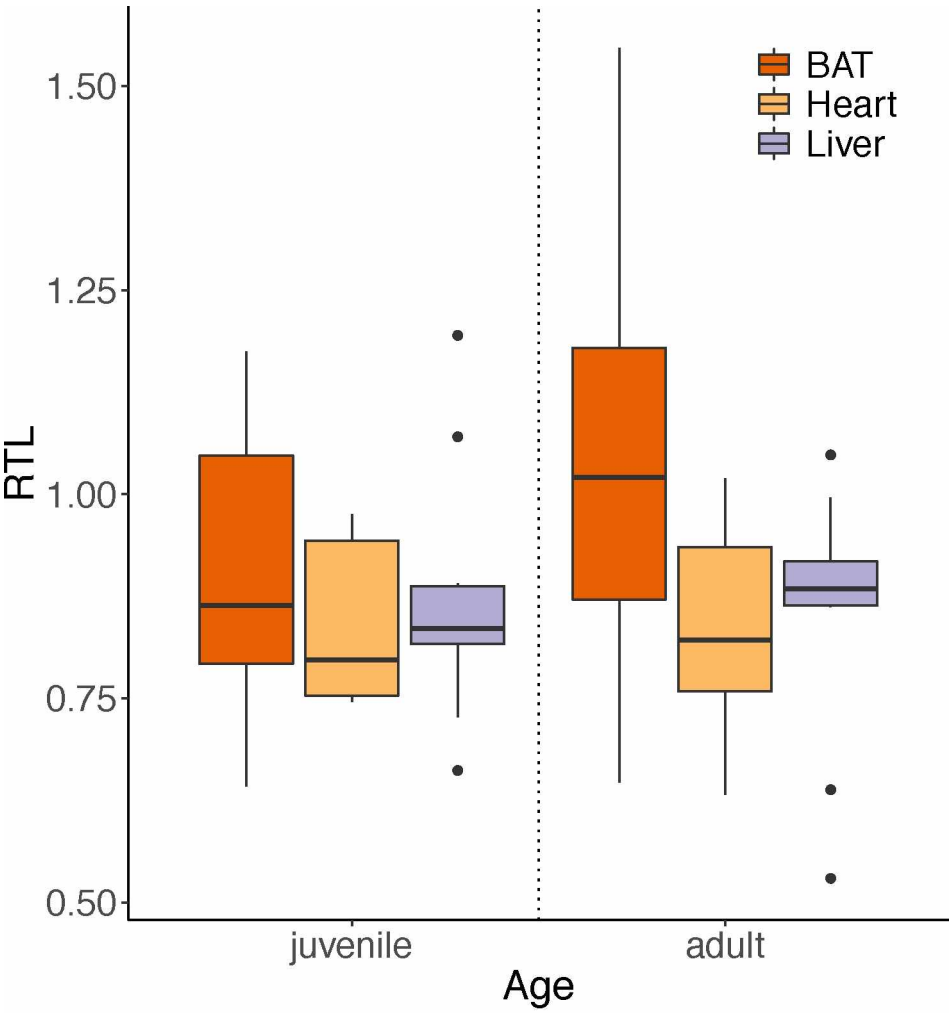

Figure 3.2. Relative telomere length (RTL) in juvenile and adult females at late hibernation. A linear mixed model was used to test for differences in RTL between tissues within an age group and within a tissue across age groups. 10 juvenile females and 10 adult females were sampled at late hibernation. There was not a significant tissue:age interaction in late hibernation females and no significant differences between planned comparisons.

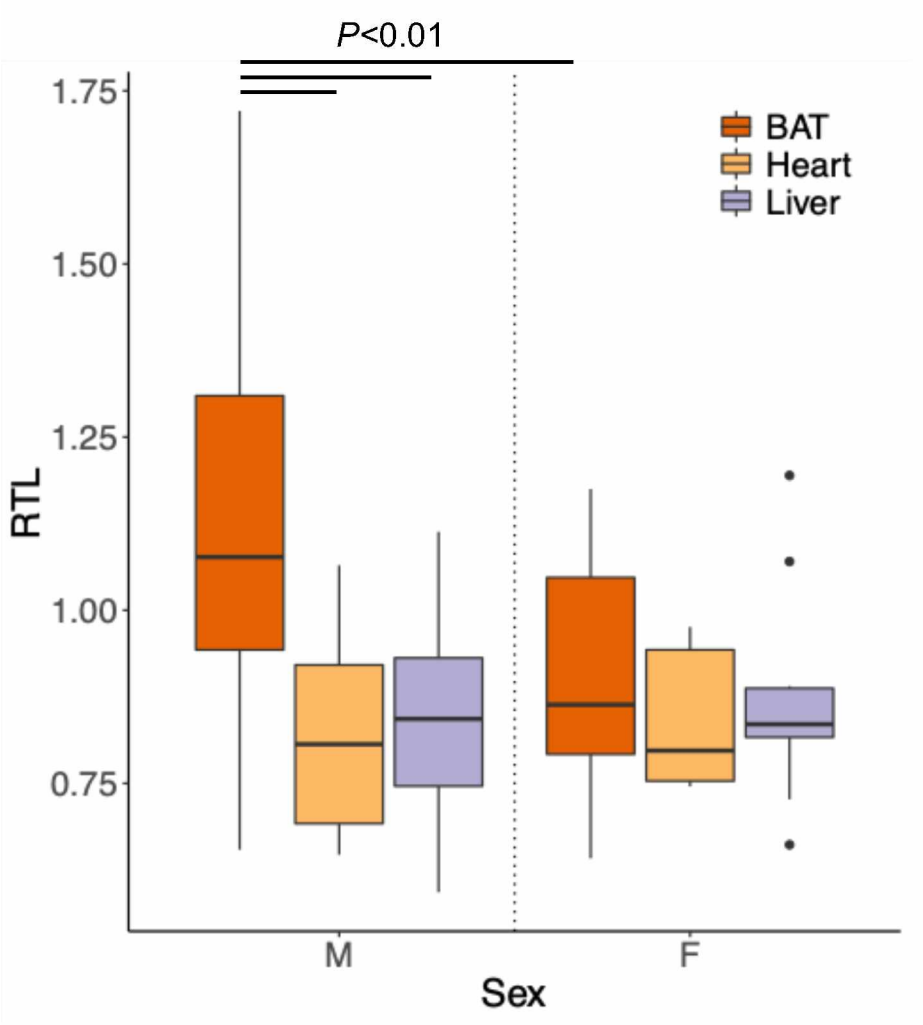

Figure 3.3. Relative telomere length (RTL) in male and female juveniles at late hibernation. Brown adipose tissue (BAT) RTL was greater than heart (P<0.001 via linear mixed model) and liver (P<0.001) in juvenile males. When comparing between sexes, BAT RTL was greater in males than in females (P=0.008). 10 juvenile females and 14 juvenile males were sampled at late hibernation. Bars indicate significant differences between groups within planned comparisons.

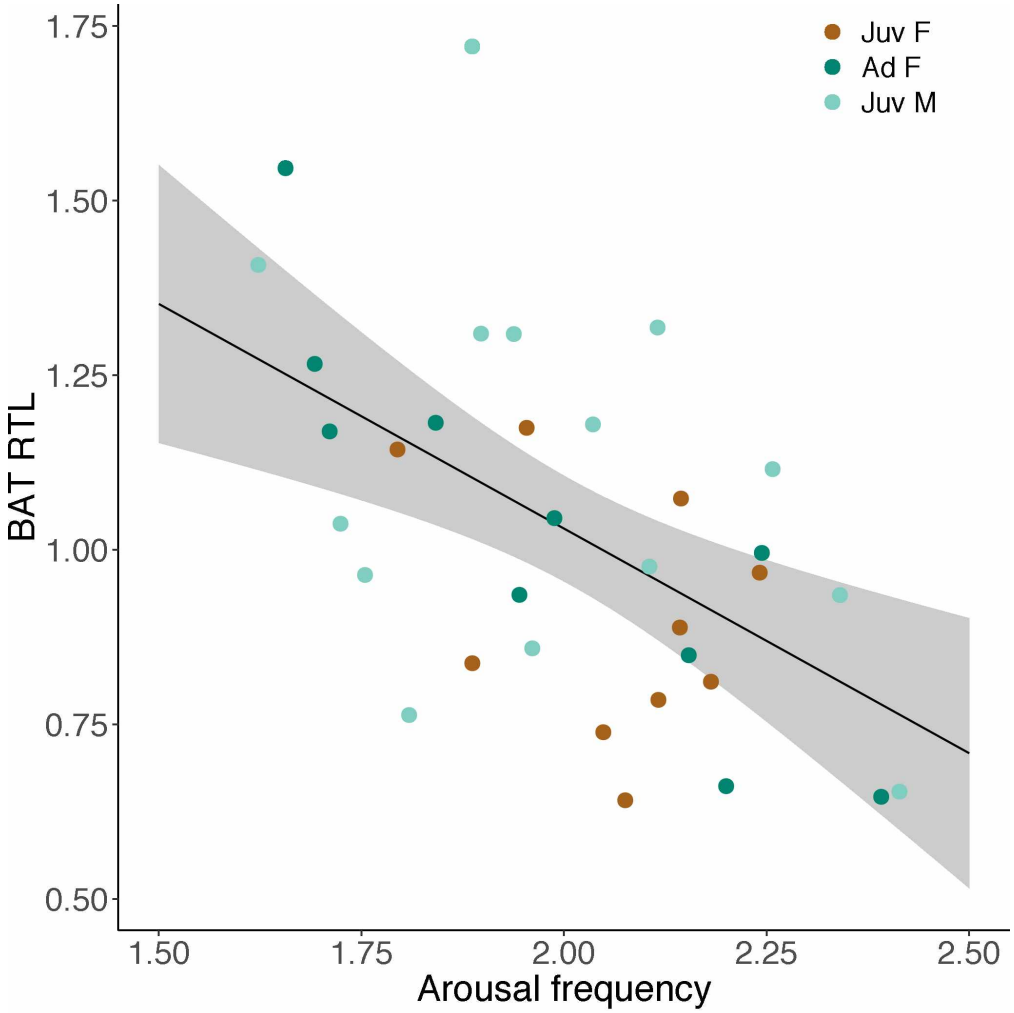

Figure 3.4. Impact of arousal frequency on relative telomere length (RTL) in brown adipose tissue (BAT). Arousal frequency had a significantly negative effect on RTL in BAT (P=0.001 via linear model) in 34 AGS at late hibernation. Juv F=juvenile female; Ad F=adult female; Juv M=juvenile male.

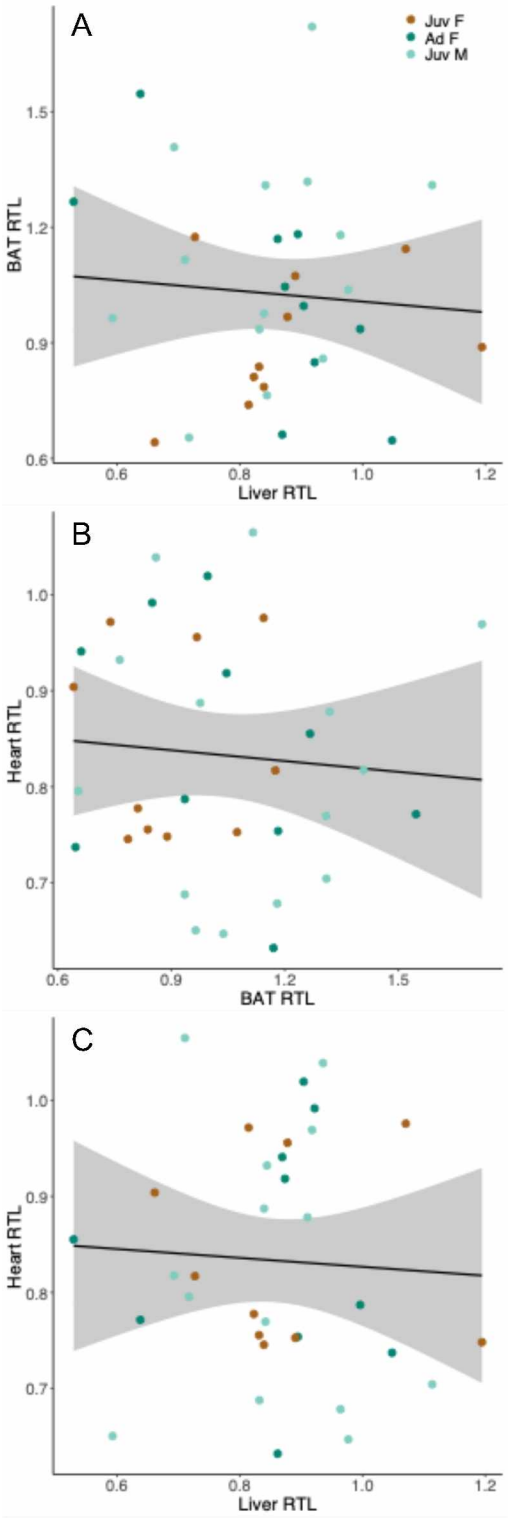

Figure 3.5. Correlation in relative telomere length (RTL) between tissues for late hibernation animals. Pairwise correlations between RTL in liver and brown adipose tissue (BAT; A), BAT and heart (B), and liver and heart (C) in 34 AGS at late hibernation. No correlations were significant. Juv F=juvenile female; Ad F=adult female; Juv M=juvenile male.

3.9 Tables

Table 3.1. Pearson's correlations of relative telomere length (RTL) between tissues for all animals. Correlation values (*r*) are reported, followed by *P*-values in parentheses. All animals (MH and LH juvenile females, LH adult females, and LH juvenile males) are included. Exclusion of MH juvenile females (to account for effect of stage) had no effect on the results.

	Liver RTL	BAT RTL	Heart RTL
Liver RTL	1	-0.18 (0.23)	-0.01 (0.95)
BAT RTL	-	1	-0.01 (0.93)
Heart RTL	-	-	1

General Conclusion

In this thesis, I have presented and discussed both basic demographics of arctic ground squirrels (AGS) in Arctic Alaska and how telomere length—a proxy for both lifespan and metabolic stress—changes in four tissues throughout hibernation. In Chapter 1, I report sex-specific lifespan, apparent annual survival, and detection for AGS from two populations north of the Brooks Range in Arctic Alaska. The oldest female recaptured from those populations was 10 years old, outliving the oldest recaptured male by four years. I attribute the longevity in females to the fact that it is a hibernating animal, and the comparatively short lifespan of the males to more time spent aboveground during the active season, engaging in aggressive territorial disputes. These behavioral differences are more pronounced in AGS than in other ground squirrel species (e.g. Wang, 1979; Michener, 1983; Healy et al., 2012), perhaps supporting a comparatively larger difference in lifespan between the sexes in this species. I also found a slightly negative effect of biologger deployment—in implanted, but not in collar-borne loggers—and show site-specific differences in survival and detection. Future demographic work could include implementing a more robust mark-recapture grid at the Atigun River site and coupling any future mark-recapture efforts with ongoing phenological and physiological research.

For Chapter 2, I measured relative telomere length (RTL) change in animals from the two sites discussed in Chapter 1 to determine if telomeres shorten throughout hibernation, an effect I proposed might occur due to thermogenic torpor bouts and intensely-energetic arousal episodes experienced by AGS in northern Alaska. In past work, it was established that temperate hibernators maintain telomere length (in peripheral tissues), and that any telomere shortening that did occur was correlated with arousal frequency (Turbill et al., 2012; Turbill et al., 2013; Giroud et al., 2014; Hoelzl et al., 2016). However, I was curious to see if this pattern existed in a species adapted to a much harsher, colder climate. I found that, in ear tissue, RTL was relatively static across hibernation, suggesting that AGS can maintain cellular integrity—as measured by telomere length—despite defending a core body temperature well above subfreezing ambient temperatures (Barnes, 1989; Karpovich et al., 2009; Richter et al., 2015). Due to difficulties in recapturing animals, I was only able to measure this effect in six individuals, so natural progressions of this work include increasing the sample size and balancing the sex ratio, as most animals measured for this component of the study were male. I also attempted to measure how RTL changes with age using a cross-sectional approach, but variability in RTL within an age cohort discouraged me from making any concrete claims about how RTL changes over time in

AGS. When using telomere length as an aging/stress biomarker with a cross-sectional approach, it is important to consider that the cohort effect may overwhelm any occurrence of telomere length change (Hall et al., 2004), in which case larger sample sizes and/or longitudinal sampling would be essential.

I identified and pursued a natural progression of past telomere-hibernator work for the study described in Chapter 3: moving beyond a single, peripheral tissue to measuring telomere length in multiple, internal, metabolically-active tissues. Sampling from captive AGS at mid- and late hibernation, and, again, using a cross-sectional approach, I measured RTL in brown adipose tissue (BAT), heart, and liver. I found that BAT RTL was dramatically shorter at late hibernation than at mid-hibernation, while RTL remain largely unchanged in heart and liver between the two timepoints. Although I did not measure this directly in my study, I attributed these patterns to reactive oxygen species (ROS) released in BAT upon non-shivering thermogenesis activation (Cannon and Nedergaard, 2004; Chouchani et al., 2016; Ballinger and Andrews, 2018). The work discussed in Chapter 3 highlighted that telomere length change in AGS is tissue-specific and is potentially driven by ROS-mediated damage to telomeres. Measuring RTL in additional tissues—such as skeletal muscle, the tissue that supports shivering thermogenesis after a critical core body temperature is attained (Brown et al., 2012)—would provide an even more holistic picture of how telomeres are changing on a per-tissue basis in this hibernating mammal. Additionally, effort could be made to implement a longitudinal study design, whereby BAT would be sampled via biopsies at various timepoints, although care should be taken that this procedure does not impact the animals' ability to hibernate naturally.

Taken together, the information presented in these chapters brings to light some long-standing unknowns about lifespan and survival patterns in AGS and also adds this extreme hibernator to the list of "unusual" model organisms (such as ectotherms; Olsson et al., 2018) that have recently diversified our understanding of telomere dynamics across taxa. In terms of AGS demographics, especially for our two well-studied populations north of the Brooks Range, the effects of biologgers on survival is especially pertinent, considering that biologging devices are being used to support conservation efforts (Wilson et al., 2015) and in understanding how organisms respond to climate change (Chmura et al., 2018). Warming in the Arctic and other climatic shifts—such as late spring snowmelt—may have an effect on AGS phenology and reproductive timing (Williams et al., 2017), and information regarding the effects of biologgers on survival and detection will be important to properly design future climate-related research.

The telomere work presented in this thesis is a logical extension of work performed by Tøien et al. (2001) and Orr et al. (2009) that investigated patterns of oxidative stress throughout hibernation in AGS. These *in vivo* studies, including this thesis, may prove useful for informing disease treatments that target oxidative damage on a cellular and tissue level (Blackburn et al., 2015), as predictable and dramatic arousals from torpor create a regular oxidative stress response, which in turn appear to shorten telomeres [see Reichert and Stier (2017) for a review on oxidative stress driving telomere shortening *in vivo*]. Future work to integrate AGS telomere dynamics with demographics should involve longitudinal tissue sampling across years to relate telomere length change to potentially shifting population processes in response to predicted Arctic climate change.

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Table A1. Model statements for apparent annual survival (ø) and detection (*p*). Sum of model weights = 0.90.

Covariates related to apparent annual survival	Covariates related to detectability	No. of parameters	$\Delta QAIC_c$	$QAIC_c$ Weight	Deviance
Age/Sex + PIT + Spring surgery + Fall surgery + Fall encounter	Age/Sex + Site + PIT	14	0	0.15	2113.15
Age/Sex + PIT + Spring surgery + Fall surgery + Fall encounter	Known age + Site + PIT	12	1.32	0.08	2118.53
Age/Sex + Site + PIT + Spring surgery +Fall surgery + Fall encounter	Age/Sex + Site + PIT	15	1.44	0.07	2112.55
Age/Sex + Collar + PIT + Spring surgery + Fall surgery + Fall encounter	Age/Sex + Site + PIT	15	1.53	0.07	2112.64
Age/Sex + PIT + Spring surgery + Fall encounter	Age/Sex + Site + PIT	13	1.93	0.06	2117.11
Age/Sex + Site + PIT + Spring surgery + Fall surgery + Fall encounter	Known age + Site + PIT	13	2.7	0.04	2117.88
Age/Sex + Collar + PIT + Spring surgery + Fall surgery + Fall encounter	Known age + Site + PIT	13	2.78	0.04	2117.96
Age/Sex + Site + Collar + PIT + Spring surgery + Fall surgery + Fall encounter	Age/Sex + Site + PIT	16	2.95	0.03	2112.03
Age/Sex + Collar + PIT + Spring surgery + Fall encounter	Age/Sex + Site + PIT	14	3.21	0.03	2116.36
Age/Sex + Spring surgery + Fall surgery + Fall encounter	Age/Sex + Site + PIT	13	3.26	0.03	2118.44
Age/Sex + PIT + Spring surgery + Fall encounter	Known age + Site + PIT	11	3.35	0.03	2122.59
Age/Sex + Spring surgery + Fall encounter	Age/Sex + Site + PIT	12	3.41	0.03	2120.62
Age/Sex + Spring surgery + Fall surgery + Fall encounter	Known age + Site + PIT	11	3.59	0.02	2122.84
Age/Sex + Site + PIT + Spring surgery + Fall encounter	Age/Sex + Site + PIT	14	3.83	0.02	2116.98
Age/Sex + Spring surgery + Fall encounter	Known age + Site + PIT	10	3.97	0.02	2125.23
Age/Sex + PIT + Fall surgery + Fall encounter	Age/Sex + Site + PIT	13	3.97	0.02	2119.15

Covariates related to apparent annual survival	Covariates related to detectability	No. of parameters	$\Delta QAIC_c$	$QAIC_c$ Weight	Deviance
Age/Sex + Collar + Spring surgery + Fall surgery + Fall encounter	Age/Sex + Site + PIT	14	4.11	0.02	2117.26
Age/Sex + Collar + Spring surgery + Fall encounter	Age/Sex + Site + PIT	13	4.13	0.02	2119.31
Age/Sex + Site + Collar + PIT + Spring surgery + Fall surgery + Fall encounter	Known age + Site + PIT	14	4.14	0.02	2117.29
Age/Sex + Site + Spring surgery + Fall surgery + Fall encounter	Age/Sex + Site + PIT	14	4.21	0.02	2117.36
Age/Sex + Collar + Spring surgery + Fall surgery + Fall encounter	Known age + Site + PIT	12	4.44	0.02	2121.65
Age/Sex + Site + Spring surgery + Fall surgery + Fall encounter	Known age + Site + PIT	12	4.52	0.02	2121.73
Age/Sex.+ Collar + PIT + Spring surgery + Fall encounter	Known age + Site + PIT	12	4.54	0.02	2121.76
Age/Sex + Collar + Spring surgery + Fall encounter	Known age + Site + PIT	11	4.68	0.01	2123.92
Age/Sex + Site + PIT + Fall surgery + Fall encounter	Age/Sex + Site + PIT	14	4.97	0.01	2118.12
Age/Sex + Site + Spring surgery + Fall encounter	Age/Sex + Site + PIT	13	4.97	0.01	2120.16
Age/Sex + Site + Collar + Spring surgery + Fall surgery + Fall encounter	Age/Sex + Site + PIT	15	5.05	0.01	2116.17
Age/Sex + Site + Collar + PIT + Spring surgery + Fall encounter	Age/Sex + Site + PIT	15	5.09	0.01	2116.2
Age/Sex + Collar + PIT + Fall surgery + Fall encounter	Age/Sex + Site + PIT	14	5.18	0.01	2118.33

Ethanol purification

Ear DNA extracts were cleaned via ethanol precipitation before performing qPCR. 50 µl of extract was combined with 150 µl of absolute ethanol, 5 µl of sodium acetate (3M, pH 5.2), and 1 µl of glycogen (all reagents from Thermo Fisher Scientific) and allowed to incubate overnight (at least 15 hours) at -20°C. Samples were centrifuged for 30 minutes at 13,400 rpm. After this initial spin, the ethanol mixture was poured off, 500 µl of 75% ethanol was added, and the samples were spun at 13,400 rpm for 10 minutes; this wash step was repeated twice. After the final ethanol removal, samples were allowed to air dry for 10 minutes until the pellet was completely dry. 50 µl TAE buffer was added to resuspend the DNA pellet.

Assessment of nucleic acid concentration and purity

All samples used in this study contained at least 14.1 ng μl^{-1} DNA with quality ratios between 1.73–1.97 for A260/A280 (a measure of protein contamination) and 1.55–2.30 for A260/A230 (a measure of phenol and/or salt contamination).

Additional qPCR specifications

Hard-shell 384-well PCR plates (thin wall, skirted, clear; Bio-Rad; Hercules, CA, USA) and MicroAmp Optical Adhesive Film (Applied Biosystems) were used for all runs.

Table A2. Sampling schedule.

Stage	Dates of collection	Tissues collected	No. animals sampled
Mid-hibernation	2 to 5 Jan 2018	Liver, heart, brown adipose	11 juvenile females
Late hibernation	1 Mar to 17 Apr 2018	Liver, heart, brown adipose	10 juvenile females, 11 adult females, 14 juvenile males

Table A3. Primer efficiencies (E) and mean coefficient of variation (mCOV) for each run.

Tissue	Telo E (%)	Telo mCOV (%)	Non-VCN E (%)	Non-VCN mCOV (%)
Brown adipose	99.8	5.9	97.6	6.4
Heart	99.3	4.9	99.5	3.6
Liver	93.6	7.0	99.9	5.7

Table A4. Correlations between hibernation parameters. Pearson's correlation tests were used for each pairwise comparison between hibernation parameters. Correlation values (*r*) and *P*-values are reported, with *P*-values in parentheses. Significant *P*-values are in bold. Only late hibernation animals (n=36) were used in this analysis.

	No. of arousals	Arousal freq.	Euthermic	Torpid	Hibernation duration
No. of arousals	1	0.39 (0.02)	0.56 (<0.01)	0.70 (<0.01)	0.77 (< 0.01)
Arousal freq.	-	1	0.15 (0.40)	-0.29 (0.10)	-0.25 (0.16)
Euthermic	-	-	1	0.21 (0.23)	0.37 (0.03)
Torpid	-	-	-	1	0.99 (< 0.01)
Hibernation duration	-	-	-	-	1